Appendix:

The Appendix includes the following items:

- Principles of Biochemistry, 2nd Edition, page 262
- Ferguson et al., Science, 4841 (239), pp. 753-759
- Lindh et al., J. Org. Chem, 1989, 54, pp. 1338-1342
- Ishizuka, Prog. Lipid Res., 36 (4), 1997, pp. 245-319
- Stryer, biochemistry, 4th Edition, page 268

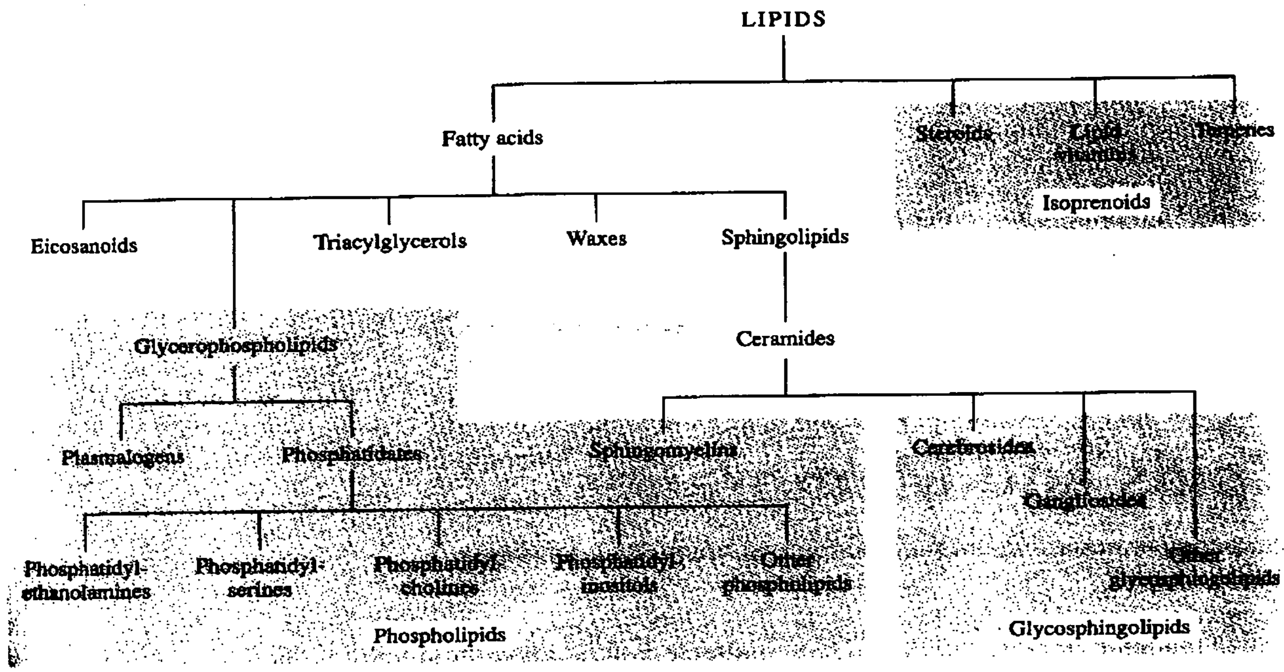

Figure 10·1
Major classes of lipids. Fatty acids are the simplest lipids. A number of other lipids either contain or are derived from fatty acids. The glycerophospholipids and the sphingomyelins contain phosphate and are classified as phospholipids. Cerebrosides and gangliosides contain sphingosine and carbohydrate and are classified as glycosphingolipids. Steroids, lipid vitamins, and terpenes are called isoprenoids because they are related to the five-carbon molecule isoprene.

In addition to diverse structures, lipids have diverse biological functions. Biological membranes contain a variety of amphipathic lipids, including glycerophospholipids and sphingolipids. In some organisms, triacylglycerols (fats and oils) function as intracellular storage molecules for metabolic energy. Fats also provide animals with thermal insulation and padding. Waxes in cell walls, exoskeletons, and skins protect the surfaces of some organisms. Some lipids have highly specialized functions. For example, the steroid hormones regulate and integrate a host of metabolic activities in animals, and eicosanoids are used to regulate blood pressure, body temperature, and smooth-muscle contraction in mammals. Gangliosides and other glycosphingolipids are located at the cell surface and may participate in cellular recognition.

10·2 Fatty Acids

More than 100 different fatty acids have been identified in microorganisms, plants, and animals. Fatty acids differ from one another in the length of their hydrocarbon tails, the degree of unsaturation (the number of carbon-carbon double bonds), and the positions of the double bonds in the chains. Some fatty acids commonly found in mammals are shown in Table 10·1. Most fatty acids have a pK_a of about 4.5 to 5.0 and are therefore ionized at physiological pH. Fatty acids can be referred to by either IUPAC (International Union of Pure and Applied Chemistry) names or common names. Common names are used for the most frequently encountered fatty acids.

The number of carbon atoms in the most abundant fatty acids ranges from 12 to 20 and is almost always even, since fatty acids are synthesized by the sequential addition of two-carbon units. (Fatty acid biosynthesis is discussed in Chapter 17.) In IUPAC nomenclature, the carboxyl carbon is labeled C-1 and the remaining

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52. Voucher specimens, where available, have been deposited at California Academy of Sciences, Golden Gate Park, San Francisco, CA. Copies of the sequences and alignments are available on written request. We acknowledge gifts of RNA or animals from R. Anderson, W. Jeffery, J. Ruderman, L. Slobodkin, and J. Valois. We thank B. Parr and J. M. Turbeville for comments on the manuscript. Supported by NSF grants BSR 85-16582 (R.A.R., N.R.P., M.T.G., and E.C.R.) and DCB 83-02149 (E.C.R.); NIH grants GM34527 (N.R.P.), HD21337 (R.A.R.), and HD16739 (E.C.R.); Office of Naval Research grant N14-86-K-0268 (G.J.O. and N.R.P.); and a MacArthur Prize Fellowship (M.T.G.).

Research Articles

Glycosyl-Phosphatidylinositol Moiety That Anchors *Trypanosoma brucei* Variant Surface Glycoprotein to the Membrane

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Two forms of protein-membrane anchor have been described for the externally disposed glycoproteins of eukaryotic plasma membranes; namely, the hydrophobic transmembrane polypeptide and the complex glycosyl-phosphatidylinositol (G-PI) moiety. The chemical structures of the major species of G-PI anchors found on a single variant surface glycoprotein (VSG) of the parasitic protozoan *Trypanosoma brucei* were determined by a combination of nuclear magnetic resonance spectroscopy, mass spectrometry, chemical modification, and exoglycosidase digestions. The G-PI anchor was found to be heterogeneous with respect to monosaccharide sequence, and several novel glycosidic linkages were present. The results are pertinent to the mechanism of the biosynthesis of G-PI anchors.

THE PARASITIC PROTOZOAN *Trypanosoma brucei* HAS A CONtinuous cell-surface coat made up of a tightly packed monolayer of variant surface glycoprotein (VSG) molecules. This VSG coat acts as a macromolecular diffusion barrier protecting the parasite from lytic host-serum components. A single trypanosome expresses only one type of VSG (variant) at a time, but has several hundred VSG genes encoding immunologically distinct VSG variants. It is the sequential expression of different VSG coats that allows the parasite to evade the host's immune response by antigenic variation (*1*). All of the different VSG variants analyzed have molecular sizes of about 55 kD and one or more asparagine glycosylation sites. Despite the lack of extensive primary amino acid sequence homology the VSG molecules are thought to share similar tertiary structures (*2*).

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From the comparison of complementary DNA (cDNA) sequences and VSG carboxyl-terminal peptide sequences (*1*) and from biosynthetic studies (*3*) it appears that VSG messenger RNA (mRNA) encodes a short COOH-terminal hydrophobic domain which is rapidly removed after polypeptide synthesis (<1 minute) and directly replaced by the addition of a glycosyl-phosphatidylinositol (G-PI) moiety. The G-PI moiety serves as the sole anchor to the membrane for the glycoprotein which then traverses the Golgi stacks to the plasma membrane. The membrane binding form of the glycoprotein (mfVSG) can be converted to a water soluble form (sVSG) by the action of an endogenous G-PI–specific phospholipase C (GPI-PLC) which removes the hydrophobic diacylglycerol group from the G-PI anchor (*4*).

The VSG G-PI anchor includes an amide linkage from the COOH-terminal amino acid α-carboxyl group to an ethanolamine residue, which bridges the VSG polypeptide to a mannose- and galactose-containing glycan (*5*). In addition, the glycan is known to contain a glucosamine residue (*6*) that is not *N*-acetylated and that is glycosidically linked to dimyristylphosphatidylinositol (*7*). We now report the complete chemical structure of the G-PI moiety present on a *T. brucei* variant surface glycoprotein.

Preparation of VSG and G-PI components. Trypanosome clones, MITat 1.4 (variant 117), of *T. brucei* strain 427 were purified from infected rat blood (*8*). Trypanosomes were also cultured in vitro with [9,10-^{3}H]myristic acid (*9*) for 1 hour before processing to produce biosynthetically labeled mfVSG. The isolation of the glycan moieties (NG) from mfVSG is described in Fig. 1. The mfVSG and sVSG glycoproteins were purified (*8*, *10*), and the intact G-PI moiety was prepared by Pronase digestion of mfVSG (*11*) and purified by HPLC (high-performance liquid chromatography) (*12*). Demyristylated G-PI (dMG-PI) was prepared from G-PI by the action of a mild base (*13*), and *N*-acetylated dMG-PI (NAc-dMG-PI) was prepared as described (*14*). The sVSG COOH-terminal glycopeptide (sCt-gp) represents the entire G-PI anchor attached to the COOH-terminal aspartic acid residue minus the dimyristylglycerol lipid moiety and was purified as described earlier (*11*).

Chemical analysis. Purified sCt-gp (800 nmol) was analyzed by one- and two-dimensional ^{1}H NMR (nuclear magnetic resonance) (Fig. 2A) and by GC-MS (gas chromatography–mass spectrometry) analysis (Tables 1 and 2). Unfractioned NG was analyzed by GC-MS compositional analysis (Table 1) and GC-MS methylation analysis before and after digestion with coffee bean α-galactosidase (Table 2). Gel-filtration on Bio-Gel P-4 (Fig. 3) resolved NG into three fractions (NG1, NG2, and NG3), which were subsequently analyzed by one-dimensional ^{1}H NMR (Fig. 2B) and GC-MS for composition (Table 1). The NG1, -2, and -3 fractions were also permethylated, purified by reversed-phase HPLC (*15*), and subjected to methylation analysis (Table 2). A sample of ^{3}H-labeled NG was also analyzed by Bio-Gel P-4 chromatography in order to determine the precise relative hydrodynamic volumes of the various neutral glycan species (Table 3).

Methylation analysis showed that all NG fractions contain three mannose residues per mole (one nonreducing terminal mannose, one 2-*O*–substituted mannose, and one 3,6-di-*O*–substituted mannose) (Table 2). Galactose residues are attached to the 3-position of the 3,6-di-*O*–substituted mannose residue since digestion with coffee bean α-galactosidase yields a single glycan species (Table 3) in which the 3,6-di-*O*–substituted mannose is quantitatively replaced by a 6-*O*–substituted mannose residue (Table 2). Treatment of unfractionated NG with a mixture of coffee bean α-galactosidase and jack bean α-mannosidase produced one major product at 1.7 glucose units as shown by Bio-Gel P-4 chromatography, which corresponds to free 2,5-anhydromannitol (2,5-AHM) (Table 3).

Purified NG1, -2, and -3, isolated by Bio-Gel P-4 chromatography, were individually treated with the Manα1-2Man–specific α-mannosidase from *Aspergillus phoenicis* (*16*). In all cases further analysis on Bio-Gel P-4 showed a reduction in hydrodynamic volume of approximately one glucose unit, consistent with the removal of one terminal α1-2–linked mannose residue common to all or most of the structures (Table 3). These data, together with interresidue nuclear

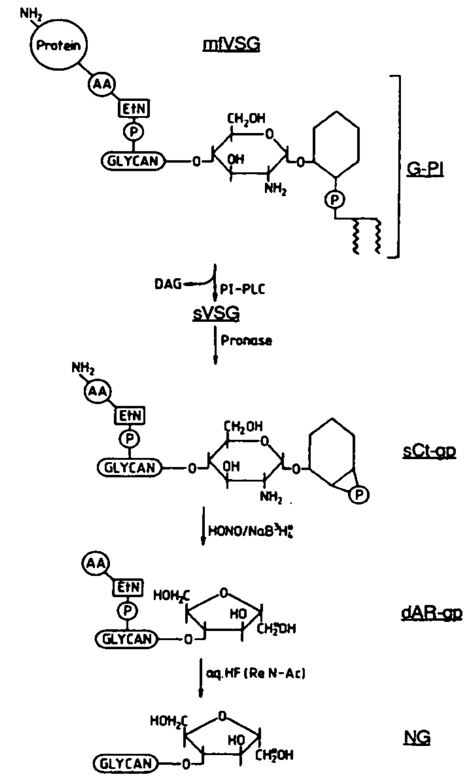
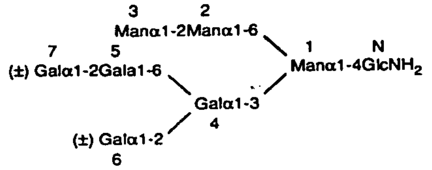


Fig. 1. Isolation of G-PI glycan fragments for structural analysis. In this study mfVSG was converted to sVSG by the action of the endogenous trypanosome GPI-PLC, which removes the diacylglycerol (DAG) moiety. Exogenous bacterial phosphatidylinositol specific phospholipase C (PI-PLC) may be used instead (*7*). The sVSG COOH-terminal glycopeptide (sCt-gp) was produced by Pronase digestion and purified (*11*). The sCt-gp (800 nmol) was dissolved in 200 μl of 0.1*M* sodium acetate, *p*H 4.0, and deaminated (200 μl of 0.5 $NaNO_2$; 2.5 hours); the material was then split into two portions of 760 and 40 nmole and reduced with $NaBH_4$ and NaB^3H_4, respectively; reduction was achieved by adding 0.26 volume of 400 m*M* boric acid, 1.26*M* NaOH, followed immediately by 1.26 volumes of either 1*M* $NaBH_4$ or 12 m*M* NaB^3H_4 (8 Ci/mmol) in 50 m*M* NaOH, boric acid buffer *p*H 11.0. Reduction was continued for 3 hours in both cases, except that excess $NaBH_4$ was added to the NaB^3H_4-treated sample after the first 80 minutes. The reduced oligosaccharitols with 2,5-anhydromannitol termini were desalted after acidification by passage through AG50X12(H^+) and methanol evaporation. Material reduced with NaB^3H_4 had radiochemical impurities removed by descending chromatography on Whatman 3 MM paper for 60 hours in 1-butanol, ethanol, water system (4:1:1). The labeled oligosaccharitols remained at the origin and were eluted with water. They were further purified by high-voltage electrophoresis on Whatman 3 MM paper for 30 minutes at 80 V/cm in pyridine, acetic acid, and water (3:1:387), *p*H 5.4. The acidic oligosaccharitols (a broad series of overlapping peaks) were eluted from the paper with water, passed through 0.1 ml of Chelex 100(Na^+) over 0.2 ml of AG50X12(H^+) and filtered through a 0.5-μm Teflon membrane. The specific activity of the deaminated and reduced glycopeptide (dAR-gp) was 1 Ci/mmol; half of this material (20 μCi) was added to the bulk $NaBH_4$ reduced material to act as labeled tracer. The dAR-gp fraction was dephosphorylated with 50 μl of 50 percent aqueous HF at 0°C for 38 hours. The sample was added to 275 μl of frozen saturated LiOH. The LiF precipitate was removed by centrifugation and washed twice with 50 μl of H_2O. The pooled supernatants were neutralized with 100 μl of saturated $NaHCO_3$; the salt was removed on a column of 0.2 ml of Chelex 100(Na^+) layered over 1 ml of AG50X12(H^+), over 0.8 ml of AG3X4(OH^-), over 0.2 ml of QAE-Sephadex A25 equilibrated with water. After filtration through a 0.5-μm Teflon filter the final yield of the neutral glycan (NG) fraction was about 70 percent. For other G-PI anchors containing *N*-acetylhexosamines a second *N*-acetylation step (RE N-Ac) is necessary. Abbreviations: AA, the COOH-terminal aspartic acid residue; EtN, ethanolamine; and P, phosphate. The hexagon represents *myo*-inositol.

Overhauser effect (NOE) measurements (Fig. 2) are consistent with the following composite structure:



The heterogeneity of the α-galactose antennae branch is evident from the composition and methylation analyses (Tables 1 and 2) and ^{1}H-NMR spectra (Fig. 2) of the sCt-gp and NG fractions. The smallest fraction from Bio-Gel P-4 gel-filtration, NG3 (Fig. 3), contains one terminal nonreducing galactose residue and one 6-O–substituted galactose residue, which defines this branch as Galα1-6Galα1-. The methylation analysis of NG2 shows both 2,6-di-O–substituted galactose and 2-O–substituted galactose present in a ratio of 7:3 (Table 2), indicating the presence of two monosaccharide sequences in this fraction. The NG2 fraction therefore contains an α1-2 linked galactose residue, which can be found linked to either the galactose-5 residue of the NG3 structure forming a linear sequence Galα1-2Galα1-6Galα- or to the internal galactose-4 residue of the NG3 structure forming a second antennae Galα1-2(Galα1-6)Galα-. The NG1 fraction has one more α1-2 linked galactose residue than NG2 and contains the sequence Galα1-2Galα1-6(Galα1-2)-Galα1- (Fig. 2).

The four major glycan structures described account for about 70 percent of the glycans. A further 15 percent can be accounted for in fractions NGA and NGB (Fig. 3) which most likely represent structures containing five or more and one or no galactose residues, respectively (Table 1). Other minor species (15 percent) present in the NG1 and NG2 fractions contain 2,3-di-O–substituted mannose (Table 2). These minor species have not been defined further.

The bridge between the G-PI moiety and the COOH-terminal aspartate residue involves an ethanolamine residue in an amide linkage to the aspartyl α-carboxyl group (*5*). Periodate oxidation was used to confirm that the ethanolamine is in a phosphodiester

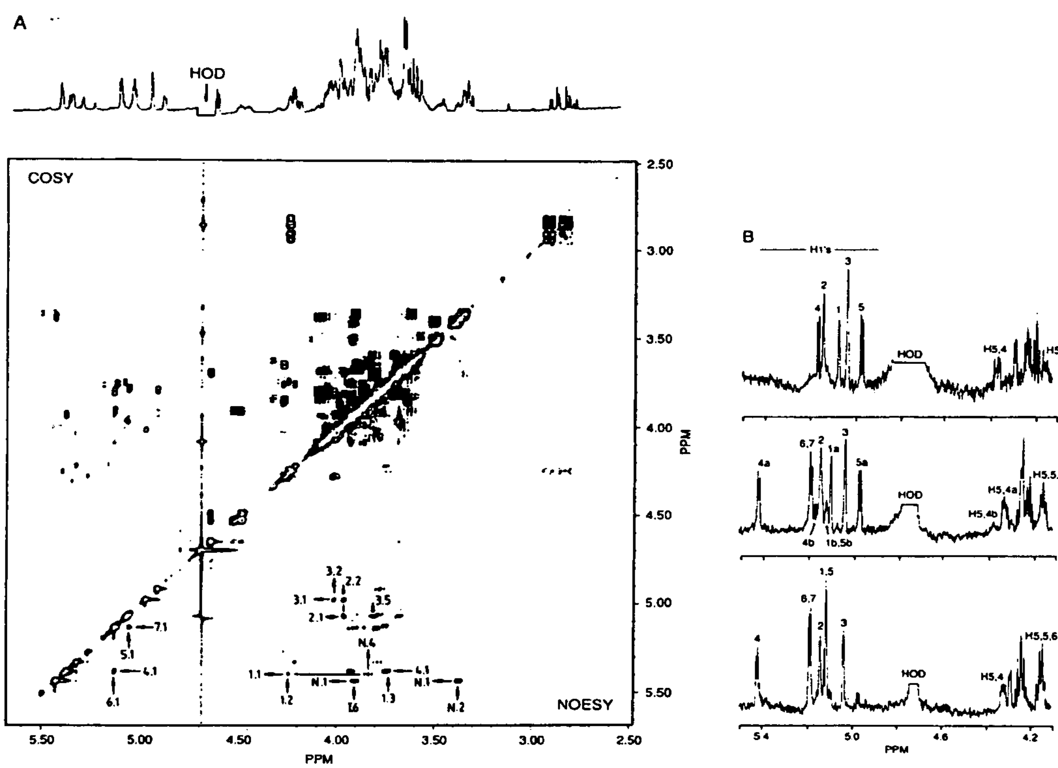


Fig. 2. ^{1}H NMR spectra of the sCt-gp and NG fractions. (**A**). The unfractionated sCt-gp structures were analyzed by high-resolution two-dimensional ^{1}H-^{1}H correlated spectroscopy (COSY), top left of figure. The spectrum was recorded as described (*38*) with a sweep width of ±1300 Hz, and 1024 data points in each dimension. For each t_1 increment 64 transients were collected. Time domain data were apodized in each dimension by means of a phase-shifted (π/12) sine-bell function. Both negative and positive contour levels are plotted with positive intensity. The one-dimensional spectrum obtained upon Fourier transformation of the data at $t_1 = 0$ is shown above. Off diagonal peaks (cross-peaks) correlate protons between which a resolved scalar (*J*) coupling exists. Through-bond–coupled protons were identified, and most of the resonances in the one-dimensional spectrum could be assigned stepwise from the resolved anomeric (H-1) protons (which resonate in the region 4.9 to 5.5 ppm). The magnitudes of the scalar (*J*) coupling between the H-1 and H-2 protons (*39*), which were measured from the splitting of the H-1 protons in one-dimensional spectra (B), were used to determine the monosaccharide residue type and the anomeric configuration. The presence of primary sequence heterogeneity in the unfractionated sCt-gp structures was evident in the one-dimensional NMR spectrum by the presence of anomeric proton resonances of less than unit intensity. The additional spectral dispersion afforded by the COSY experiment allowed the connectivity networks for each residue to be traced separately, and four major species could be identified. After resonance assignment, all through-space connectivities between proximal protons (<4 Å distant) were mapped by use of ^{1}H-^{1}H nuclear Overhauser effect spectoscopy (NOESY) as described previously (*38*), bottom right of figure. The spectrum was recorded under identical conditions to the COSY experiment with a mixing time of 500 msec. The cross-peaks in NOESY, for this particular mixing time, generate qualitative through-space connectivities within the molecule. Connectivities were assigned to specific protons by means of the resonance assignments derived from COSY. Intraresidue and interresidue through-space connectivities were defined for each structure, and the primary sequences of the monosaccharide residues were determined stepwise along the molecule with the use of these connectivities and methylation analysis data. Representative connectivities are shown for the largest glycan structure, where the cross-peak label abbreviations correspond with the residue descriptors (described in the text) followed by the ring proton number. Cross-peaks labeled I are due to inositol. (**B**) One-dimensional ^{1}H NMR spectra of major neutral glycan fractions NG3 (top panel), NG2 (middle panel), and NG1 (lower panel). Each spectrum was recorded with a sweep width of ±1300 Hz, 16,384 data points, and 200 transients. The chemical shift axis is referenced to acetone, $\delta = 2.225$ ppm at 30°C. The proposed resonance assignments are deduced from the magnitudes of J_{12} (*39*) and from the characteristic chemical shifts observed between fractions, which are due to differences in primary sequence. The notation corresponds with that described earlier. In the middle panel, the a and b for residues 1, 4, and 5 correspond to each of the two structures found in fraction NG2.

linkage to the 6-position of either the terminal or the penultimate 2-O-substituted mannose as suggested by the presence of mannose-6-phosphate in the methanolyzates of sCt-gp (Table 1). In either case, periodate oxidation would cleave the mannose residue between C-3 and C-4 to yield a [1-^{2}H]glycerol group after reduction with NaB^2H_4 (derived from mannose C-4, C-5, and C-6), with the phosphorylethanolamine–aspartic acid group remaining attached to the mannose-6-hydroxyl (Fig. 4A). In addition, acid hydrolysis was used on the periodate-oxidized and NaB^2H_4-reduced demyristylated

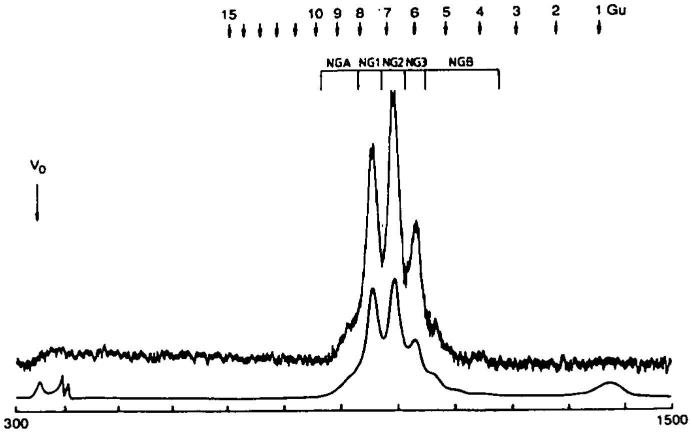


Fig. 3. Bio-Gel P-4 chromatography of the neutral glycan (NG) fraction. The ^{3}H-labeled neutral glycan (NG) fraction of the mfVSG G-PI anchor (Fig. 1) was fractionated on two columns in series (1.5 × 100 cm each) packed with Bio-Gel P-4 (−400 mesh) held at 55°C and eluted with water at 0.2 ml/min. The eluate was monitored for radioactivity (Berthold LB503 radioactivity flow monitor) and refractive index (Erma ERC7510 monitor) before collection in 0.5-ml fractions. The upper broad trace represents radioactivity and the lower smooth trace refractive index. The major peaks NG1, NG2, and NG3 and the minor flanking regions NA and NB were pooled separately for analysis. The numbers at the top represent the elution positions of dextran oligomers (number of glucose units) determined in a separate experiment.

Table 1. Compositional analyses. All analyses were performed in the presence of a *scyllo*-inositol internal standard. Samples were subjected to methanolysis (50 μl of 0.5*M* HCl, 20 percent methyl acetate in dry methanol, 20 hours, 70°C), *N*-acetylation (by the subsequent addition of 10 μl of pyridine and 5 μl of acetic anhydride, 30 minutes at room temperature) and trimethylsilylation (TMS) with 15 μl of Sil-A (Sigma) after drying. The TMS derivatives were analyzed with a Hewlett-Packard 5996 GC-MS equipped with an open-split interface for simultaneous mass spectral identification and flame ionization detector (FID) quantitation. Spectra were recorded by electron-impact (70 eV). A fused silica bonded phase column 25 m × 0.32 mm (RSL150, Alltech) with He as carrier gas at 3 ml/min was used in the GC system. Direct on-column injection was used with a temperature program of 140°C (2 minutes) and a linear gradient to 250°C at 6° per minute, held for 15 minutes. All figures shown here and in Table 2 and Fig. 5 are means of at least two analyses and are subject to experimental error of ±15 percent. ND, not determined.

Material	Proportion (%)*	Molar ratio				
		2,5-AHM	Man	Gal	Man-6-P†	*myo*-inositol
sCt-gp		0.0	2.0	3.7	0.5	0.0‡
dAR-gp		1.0	2.0	4.0	ND	0.0‡
NG	100	1.0	2.8	3.7		0.7
Bio-Gel P-4 fractions§						
NGA	7	0.9	3.0	5.4		
NG1	30	1.0	3.0	3.9		
NG2	40	1.1	3.0	3.0		
NG3	15	0.9	3.0	2.0		
NGB	8	0.6	3.0	1.5		

*Percentage of the total ^{3}H label in each fraction. †Analyses for mannose-6-phosphate were as described above except that after trimethylsilylation the products were dried, redissolved in 10 percent methanol in ether (0°C, 10 minutes) and treated twice with an equal volume of ether saturated with diazomethane at 0°. The dimethyl phosphate derivatives were dried, redissolved in Sil A (Sigma), and analyzed as described, above. ‡Absent in these analyses due to the acid stability of the inositol-phosphate and glucosamine-inositol bonds to methanolysis (*7*). §See legend to Fig. 3.

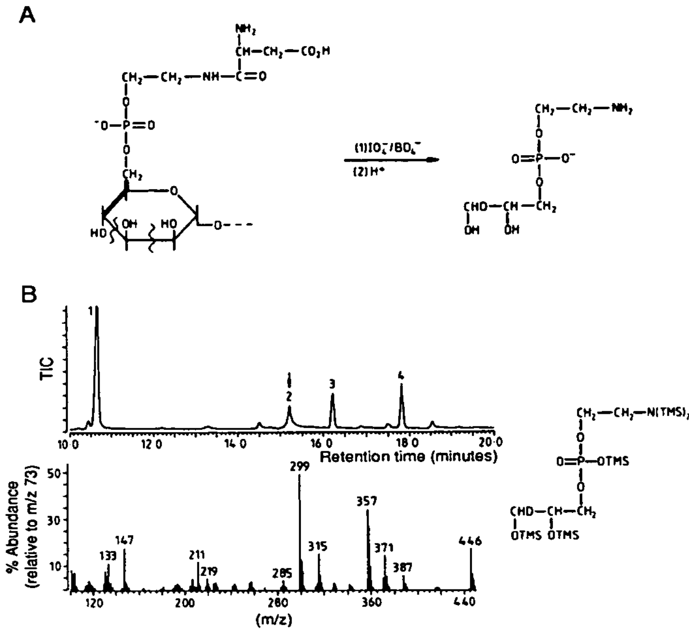
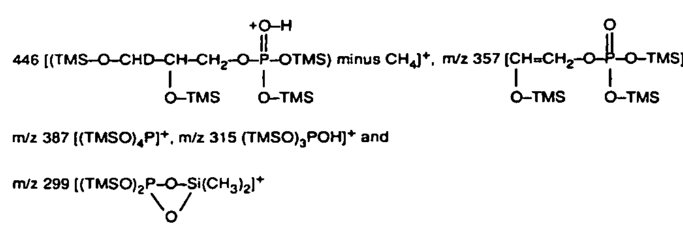


Fig. 4. Periodate oxidation of the mannose-6-phosphorylethanolamine bridge. (**A**) The wavy lines indicate the sites of periodate oxidation cleavage. The bold lines indicate the regions of the mannose carbon backbone which survive oxidation. Periodate oxidation was performed on 120 nmol of dMG-PI in 30 m*M* $NaIO_4$, 200 m*M* sodium acetate buffer, *p*H 4.5, containing 0.1 percent 1-propanol as radical scavenger. Oxidation was performed at twofold molar excess of periodate over theoretical oxidation sites (estimated as 13 mol per mole of dMG-PI) at 4°C in the dark. The reaction was followed by monitoring the decrease in absorbance due to the IO_4^- ion at 223 nm after dilution (1:300) of small portions with water. Oxidation reached a maximum by about 15 hours. After 20 hours, the reaction was stopped by making the solution 1 percent (by volume) with respect to ethylene glycol. After 30 minutes, the products were reduced by the addition of an equal volume of 1*M* NaB^2H_4 in 1*M* NH_4OH (final *p*H 9.8). After 3 hours, the excess NaB^2H_4 was destroyed with acetic acid, and the mixture was desalted by passage through AG50X12(H^+), followed by repeated evaporation with acidified methanol. (**B**) Periodate oxidized and borodeuteride-reduced dMG-PI was hydrolyzed (2*M* HCl, 100°C, 4 hours), dried, and trimethylsilylated with the reagent described in (*40*). The products were analyzed by GC-MS as described in the legend to Table 1 except that the GC program was 100°C (held for 2 minutes) to 260°C at 6° per minute. The total ion chromatogram (upper panel) shows the presence of [1-^{2}H]erythritol-TMS_4 (peak 1) and α- and β-mannose-TMS_5 (peaks 3 and 4). Peak 2 had the same retention time and mass spectrum (lower panel) as the TMS derivative of authentic glycerophosphoryl ethanolamine generated by deacylation of phosphatidylethanolamine (*13*). The spectrum shows the ions characteristic of a [1-^{2}H]glycerophosphoryl derivative at *m/z*.



G-PI (dMG-PI) to exploit the relatively acid-stable phosphodiester linkage while quantitatively removing the acid-labile aspartic acid group. After trimethylsilylation of the above, [1-^{2}H]glycerophosphoryl ethanolamine was identified by GC-MS (Fig. 4B) and confirmed the presence of ethanolamine phosphate linked to the 6-position of mannose.

Susceptibility to digestion by jack bean α-mannosidase was used to probe the position of the ethanolamine phosphate bridge. From the substrate specificity of jack bean α-mannosidase the terminal mannose residue should be resistant to digestion only if this residue is the site of the ethanolamine phosphate linkage. The deaminated and reduced glycopeptide (dAR-gp) fraction, which still contains the aspartic acid–ethanolamine phosphate bridge (Fig. 1), was first treated with coffee bean α-galactosidase to remove the α-galactosyl branch heterogeneity. Subsequent cold aqueous hydrogen fluoride dephosphorylation produced one major elution product with 4.2 glucose units on Bio-Gel P-4, corresponding to the Manα1-2Manα1-6Manα1→4,2,5-AHM region (Table 3). Digestion of this neutral structure with jack bean α-mannosidase converted this peak to one at 1.7 glucose units (2,5-anhydromannitol). In contrast, digestion of the agalactosyl dAR-gp with the α-mannosidase prior to HF dephosphorylation and Bio-Gel P-4 analysis failed to remove any of the α-mannose residues. This result suggests that it is the terminal α-mannose residue that is linked to the ethanolamine phosphate bridge.

Periodate oxidation was also used to probe the mannose→glucosamine→inositol monosaccharide sequence. The predicted sites of periodate oxidation of the demyristylated G-PI (dMG-PI) fraction are shown in Fig. 5A; the hexose residues not shown have free hydroxyl groups at C-3 and C-4 and will be oxidized and reduced to [1-^{2}H]glycerol. The periodate-oxidized and NaB^2H_4-reduced dMG-PI was hydrolyzed, treated with alkaline phosphatase, and analyzed by GC-MS after peracetylation of the products (Fig. 5B). The three predicted major products were found: [1-^{2}H]glycerol, an intact mannose residue (derived from the protected 3,6-di-*O*–substituted mannose branch-point residue), and [1-^{2}H]erythritol (derived from the glucosamine residue) (Fig. 5B).

^{1}H NMR data suggest that the glucosamine is linked α1–6 to the *myo*-inositol residue as evidenced by a NOE linking $GlcNH_2$ H-1 to inositol H-6 (Fig. 2A). The predicted oxidation and reduction product of the inositol ring is therefore [1,4-di-^{2}H]threitol (derived from carbons 1, 2, 5, and 6). However this component was observed only in trace amounts in the products of periodate oxidation of the dMG-PI sample described above. On the basis of the observations of (*17*) it is likely that this product is lost because of the rapid overoxidation of the glucosamine residue from C-2, and onward through to the inositol ring. *N*-Acetylation of the glucosamine residue present on dMG-PI renders the residue resistant to periodate oxidation (Fig. 5A). The NAc-dMG-PI was treated as above for dMG-PI, and Fig. 5C shows the recovery of [1-^{2}H]glycerol, an intact protected mannose residue as before, and intact *N*-acetylglucosamine in place of the [1-^{2}H]erythritol. In addition, the predicted [1,4-di-^{2}H]threitol product is now observed in good yield. A di-deuterated tetritol can only arise from the prior oxidation of a polyol (in this case *myo*-inositol) and not from a hexose or hexosamine, which can only generate a monodeuterated tetritol. From the stereochemical arrangement of the *myo*-inositol ring, threitol must be derived from carbons at positions 1, 2, 5, and 6. This implies that the inositol ring was originally substituted at C-1 and C-6. The phosphatide group is known to be at C-1 (*7*), thus leaving only C-6 for glycosidic substitution by the glucosamine residue.

Table 2. Methylation analyses. In all cases samples were permethylated according to (*35*). Permethylated glycans were hydrolyzed (2.5 hours at 80°C in 100 μl of 0.25*M* H_2SO_4, 93 percent aqueous acetic acid), applied to a 0.5-ml column of AG3X4 (acetate form) and eluted with five column volumes of 50 percent aqueous methanol. Following repeated evaporation with toluene the hydrolyzates were reduced with NaB^2H_4 (200 μl; 10 mg/ml; 3 hours) with sonication. Excess NaB^2H_4 was destroyed with acetic acid, and boric acid was removed by repeated evaporation (five times) with 0.2 ml of methanol. The products were dried and acetylated with 250 μl of acetic anhydride (100°C, 2.5 hours). The acetic anhydride was removed at reduced pressure. The resulting partially methylated alditol acetates (PMAA's) were recovered by partitioning between CH_2Cl_2 and water. The CH_2Cl_2 phase was concentrated to about 20 μl, and 2-μl portions were analyzed by GC-MS (Supelcowax 10 column, 30 m by 0.32 mm; Supelco) with He as carrier gas (2.5 ml/min) and direct on-column injection. The temperature program was started at 90°C (1 minute) followed by a linear gradient to 140°C at 30°C per minute, and then to 250°C at 5°C per minute, and held for 15 minutes. The PMAA's were identified by their characteristic mass spectra and retention times, and were quantified by their flame-ionization detector response with semiempirical molar correction factors (*36*).

PMAA	Origin	sCt-gp*	NG	α-Galactosidase treated NG	Subfractions		
					NG1	NG2	NG3
2,5-Anhydro mannitol†							
(1,3,6-tri-*O*-methyl-4-*O*-acetyl)	4-*O*-subs. 2,5-AHM	0.0	†	†	†	†	†
Mannitol							
(2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl)	Terminal-Man	0.6	1.0	1.0	1.0	1.0	1.0
(3,4,6,-tri-*O*-methyl-1,2,5-tri-*O*-acetyl)	2-*O*-subs. Man	1.0	1.0	1.0	1.2	1.0	1.3
(2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetyl)	6-*O*-subs. Man	0.0	0.0	0.9	0	0.0	0.0
(2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetyl)	3,6-*O*-disubs. Man	0.9	0.9	0.0	0.9	0.8	1.1
(4,6-di-*O*-methyl-1,2,3,6-tetra-*O*-acetyl)	2,3-*O*-disubs. Man	0.2	0.2	0.0	0.1	0.2	0.0
Galactitol							
(2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl)	Terminal-Gal	2.0	1.7	[0.8]‡	[illegible]	1.7	0.9
(2,3,5,6-tetra-*O*-methyl-1,4 di-*O*-acetyl)		0.0	0.0	[0.9]‡	[illegible]	0.0	0.0
(3,4,6,-tri-*O*-methyl-1,2,5-tri-*O*-acetyl)	2-*O*-subs. Gal	0.3	0.4	0.0	[illegible]	0.3	0.0
(2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetyl)	6-*O*-subs. Gal	0.4	0.4	0.0	0.0	0.3	1.1
(3,4-di-*O*-methyl-1,2,5,6-tetra-*O*-acetyl)	2,6-*O*-disubs. Gal	1.0	0.6	0.0	1.0	0.7	0.0
2-*N*-Methylacetamido-2-deoxyglucitol							
(3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl)	4-*O*-subs. GlcNAc	0.6*	—	—	—	—	—

*The intact sCt-gp sample (35 nmol) was subjected to methylation analysis following *N*-acetylation in 100 μl of saturated $NaHCO_3$ with 5 μl of acetic anhydride at 0°C. The *N*-acetylated sCt-gp was passed through AG50X12(H^+) and dried in the presence of a tenfold molar excess of triethylamine to produce demethyl sulfoxide soluble triethylamine salts of the sCt-gp for permethylation. The resulting PMAA's were analyzed by GC-MS with a bonded OV-17 column (24 m by 0.32 mm, RSL-300, Alltech Associates) with a temperature program of 90°C (1 minute) to 200°C at 30°C per minute, held for 30 minutes. †1,3,6-tri-*O*-methyl-4-*O*-acetyl-2,5-anhydromannitol was detected in low and variable yield due to its high volatility. ‡The square brackets indicate the presence of free galactose in the α-galactosidase digest.

Features of the G-PI anchor. The biochemistry of glycosyl-phosphatidylinositol (G-PI)–anchored proteins has been reviewed (*18*). Of these examples, rat Thy-1 glycoprotein and *Trypanosoma brucei* variant surface glycoprotein (VSG) are the best characterized in terms of the structure of their G-PI anchors (*7*, *19*). The trypanosome is a good model system for the study of the structure and biosynthesis of G-PI anchors since the VSG coat represents about 10 percent of the total cell protein and can be readily purified in tens of milligrams.

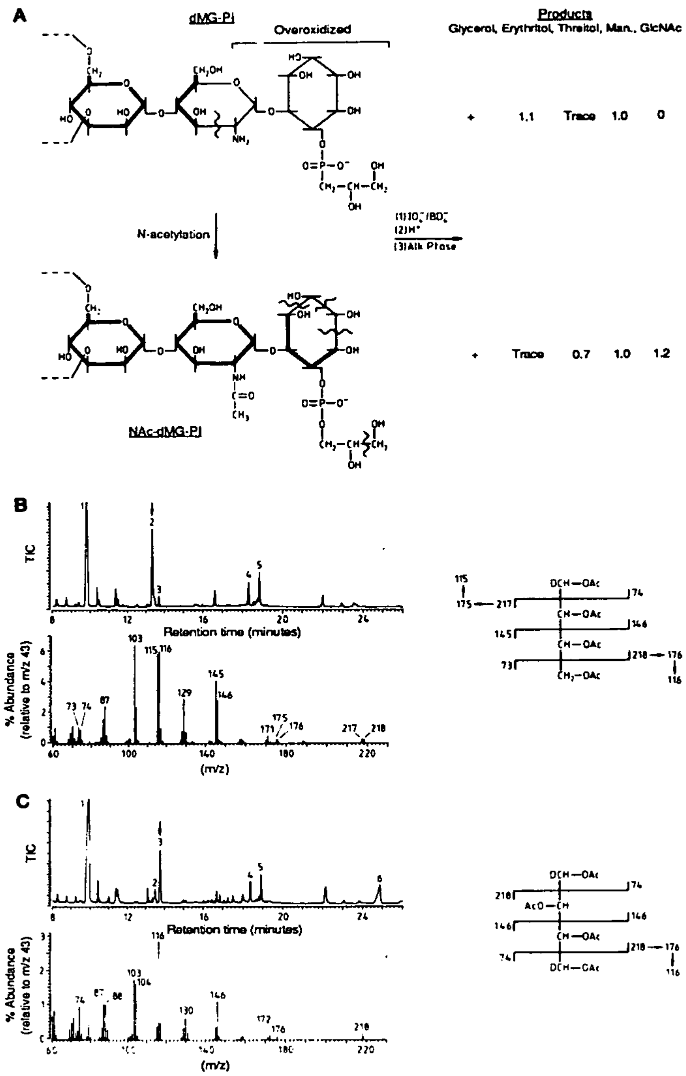


Fig. 5. Periodate oxidation of the Manα1-4GlcNH$_2$$\alpha$1-6 myo-inositol core region. Samples (120 nmol) of dMG-PI and its *N*-acetylated form, NAc-dMG-PI, were periodate oxidized, and NaB^{2}H$_4$ reduced (Fig. 4). The desalted products were hydrolyzed (2*M* HCl, 100°C, 3 hours), dried, and treated with 5 units of bovine alkaline phosphatase in 15 μl of 66 m*M* NH_4HCO_3 for 24 hours at 37°C. After passage through AG50X12(H$^+$), the products were dried and per-*O*-acetylated with 15 μl of acetic anhydride and pyridine (1:1) at 100°C for 30 minutes. Samples (1 to 2 μl) were analyzed by GC-MS. The Supelcowax 10 column was used with a temperature program of 100°C (held for 2 minutes) to 260°C at 10°C per minute, held for 15 minutes. The products were quantitated from their flame ionization detector signals with the use of empirically determined relative response and hydrolytic destruction correction factors. (**A**) The periodate oxidation sites are shown by waved lines. The bold lines represent the carbon-carbon bonds resistant to oxidation. (**B**) The GC-MS analysis of the periodate-treated dMG-PI sample showing the total ion current chromatogram (upper panel) and the mass spectrum of peak 2, [1-^{2}H]erythritol (lower panel). (**C**) The GC-MS analysis of the periodate-treated NAc-dMG-PI sample showing the total ion current chromatogram (upper panel) and the mass spectrum of peak 3, [1,4-di-^{2}H]threitol (lower panel). The other numbered peaks were identified as the acetate derivatives of [1-^{2}H]glycerol, peak 1; α- and β-mannose, peaks 4 and 5; and *N*-acetylglucosamine, peak 6.

The VSG G-PI anchor as described above contains several novel structural features (Fig. 6), particularly in the glycan region. The monosaccharide sequences Galα1-2Gal, Galα1-6Gal, Galα1-3Man, Manα1-4GlcNH$_2$, and GlcNH$_2$$\alpha$1-6*myo*-inositol apparently have not been previously described in eukaryotic glycoproteins.

Nonacetylated glucosamine bearing a free amino group appears to be a common feature of G-PI anchors and has been found in rat Thy-1 (*19*, *20*), human erythrocyte acetylcholinesterase (AChE) (*21*), and human erythrocyte decay accelerating factor (DAF) (*22*). Furthermore a GlcNH$_2$-inositol linkage has been inferred for *Torpedo* AChE and human placental and bovine intestinal alkaline phosphatase by deamination studies (*23*). The ethanolamine bridge between the COOH-terminal amino acid and the G-PI anchor is also conserved in Thy-1 (*19*) and human erythrocyte AChE (*21*). However, these examples (Thy-1, AChE) and DAF also contain additional ethanolamine residues with unsubstituted amino groups (*20*–*22*). Similarities in the glycan structures of several G-PI anchors are also suggested by immunological studies. A cross-reacting determinant (CRD) is present in the G-PI glycans of different sVSG's (*24*). These antibodies to CRD (anti-CRD) have also bound to PI-PLC–solubilized *Leishmania* antigen, DAF, and *Torpedo* and human erythrocyte AChE (*25*). The full extent of the structural homology indicated by anti-CRD binding must await definition of the CRD epitope. Clearly, some differences in the G-PI glycans do occur since the Thy-1 anchor contains *N*-acetylgalactosamine and no galactose (*19*). Nevertheless, it is possible that the mannose branch (Fig. 6) is conserved since the Thy-1 anchor contains comparable amounts of mannose (*19*).

A number of other glycosylated phosphoinositides exist in nature, although not linked to protein. These include the mannosylphosphatidylinositols of mycobacteria, the glycosylated inositol phosphoceramides of plants and yeasts (*26*), and a complex acidic lipophosphoglycan of *Leishmania donovani*. The last mentioned resembles a G-PI anchor in that it is a glycosyl-(lysoalkyl)-phosphatidylinositol–linked to a series of repeating disaccharide units rather than to protein (*27*). The phosphatidylinositol-glycans reported to be the precursors of second messengers for some of the actions of insulin also contain a GlcNH$_2$-inositol linkage and appear to be structurally related to G-PI anchors (*28*).

The signal for G-PI addition to nascent polypeptide resides in a short hydrophobic COOH-terminal peptide sequence which is removed and replaced by the G-PI anchor (*18*). The rapid kinetics of G-PI addition to VSG strongly suggests that the anchor is added as a unit (en bloc) to the polypeptide, and candidate precursor G-PI molecules have been identified and characterized (*29*, *30*). Whereas the G-PI biosynthetic pathway is not well understood, the composite structure (Fig. 6) indicates that it will involve several novel enzymes. However, studies on the class E thymoma mutant suggest that, for Thy-1, dolichol-phosphoryl-mannose (Dol-P-Man) may be involved in G-PI synthesis (*31*). Since enzymes that catalyze the formation of Manα1-2Man and Manα1-6Man linkages (Fig. 6) from a Dol-P-Man donor are known in the dolichol cycle of *N*-glycosylation (*32*), it is possible that G-PI biosynthesis may share one or both of these enzymes. The novel α-galactose antennae of the VSG anchor is probably added after transfer of G-PI to VSG polypeptide since the putative G-PI precursor contains only mannose (*30*). The extent and heterogeneity of galactosylation found in an individual VSG may reflect steric constraints of the attached

polypeptide to the approach of the relevant α-galactosyltransferases since there is a correlation between the degree of galactosylation with VSG subclass (*24*). These subclasses are based on COOH-terminal peptide homology where class 1 VSG's contain about 4 mol of galactose per mole of G-PI, class 2 VSG's contain eight galactose residues, and class 3 VSG's do not contain any galactose residues.

At present the functional significance of using a G-PI anchor rather than a transmembrane amino acid sequence is unknown. Possible advantages may include conservation of space in the lipid bilayer, a higher degree of lateral mobility in the bilayer, as found for Thy-1 (*33*), and the potential to be solubilized by endogenous anchor-specific phospholipases (*4*).

Note added in proof: Since submission of this article some structural details of another VSG G–PI anchor have been described (*34*).

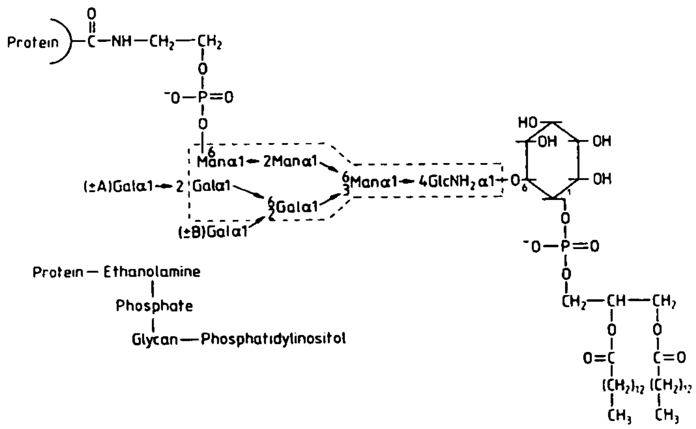
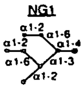
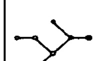
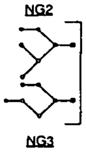
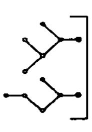
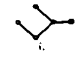


Fig. 6. Complete primary structure of the G-PI anchor of VSG variant 117. The area enclosed by the broken line represents the common region of the glycan moiety which is further substituted with one or two α1-2 linked galactose residues, residues A and B. The four structures represented account for 70 percent of all the structures present on VSG variant 117 (25 percent plus A and B, 22 percent minus A plus B, 8 percent plus A minus B, and 15 percent minus A and B).

Table 3. Exoglycosidase studies. In the left-hand column mannose residues are represented by ●, galactose by ○, and 2,5-anhydro-mannitol by ■. The glycosidic linkages are shown in the NG1 structure. The various structures were generated by exoglycosidase digestion of the whole NG fraction (NG1, -2, and -3) or individual NG substrates (NG1, NG2, or NG3). All enzyme digests were performed on NaB^3H_4-reduced glycans (Fig. 1) at substrate concentrations of 50 μ*M* in 0.1*M* sodium acetate, *p*H 5.0, for 18 hours at 37°C. The enzymes used were *Aspergillus phoenicis* Manα1-2Man specific (*16*) α-mannosidase (Apα1-2Man), 20 μg/ml; coffee bean α-galactosidase (CBα-Gal), 30 unit/ml; and jack bean α-mannosidase (JBα-Man), 60 unit/ml. The size (relative hydrodynamic volume) of each species was determined by Bio-Gel P-4 chromatography as described in Fig. 3 except that the samples were coinjected with a set of glucose oligomers (200 μg of dextran hydrolyzate). The sizes of the radioactive glycans (1 to 3 μCi) were determined by interpolation of their elution positions between the glucose oligomer elution positions as described in (*37*).

Structure	Substrate	Apα1-2Man	CBα-Gal	JBα-Man	Size (glucose units)	Structure	Substrate	Apα1-2Man	CBα-Gal	JBα-Man	Size (glucose units)
NG1 	NG1	–	–	–	7.6		NG1	+	–	–	6.6
NG2 	NG2	–	–	–	6.8		NG2	+	–	–	5.7
NG3 	NG3	–	–	–	6.1		NG3	+	–	–	5.0
	NG1,2,3	–	+	–	4.2		NG1,2,3	–	+	+	1.7

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12. The Pronase digest (2 ml) was acidified with 20 μl of acetic acid, 0.5 ml of 2-propanol was added, and the mixture was centrifuged. The supernatant was applied to a 5-μm Ro-Sil C8 column (0.46 by 25 cm, Alltech Associates) in sequential 0.5-ml injections and eluted with a gradient of 20 to 100 percent 2-propanol in 0.05 percent aqueous trifluoroacetic acid over 80 minutes at 0.8 ml/min. The G-PI moiety was detected by following a [^{3}H]myristic acid label and was eluted between 53 and 77 percent 2-propanol. The G-PI fractions were dried by rotary evaporation at 25°C and stored in 56 percent 2-propanol at −20°C.
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14. dMG-PI (120 nmol) was *N*-acetylated in 100 μl of saturated $NaHCO_3$ by three additions of 2.5 μl of acetic anhydride 10 minutes apart. The product was desalted by passage through AG50X12(H^+) and evaporation with toluene.
15. Permethylated glycans were purified on a 5-μm Ro-Sil C18 column (0.46 by 25 cm, Alltech Associates) eluted with a gradient of acetonitrile in H_2O, 25 to 65 percent, over 60 minutes at 1 ml/min. Permethylated NG1, NG2, and NG3 fractions eluted at 49, 46, and 42 percent acetonitrile, respectively.
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Anal. Calcd for $C_{21}H_{24}O_4{\cdot}2H_2O$: C, 67.00; H, 7.49. Found: C, 67.33; H, 6.78.

20-Acetoxy-3,11-dioxo-1,4-*trans*-17(20)-pregnatrien-21-al (7e-*trans*): small prisms from ethanol; mp 207–210 °C; $[\alpha]_D$ +147°; λ_{max} 244 nm; ϵ 30 500; IR 1768 and 1200 (enol acetate), 1710 (11-ketone), 1690–1610 cm^{-1} (multiple peaks, conjugated carbonyls); CI-MS m/z 383 (M^+ + 1, 100), 341 (M^+ + 1 − CH_3COH, 13). NMR spectra were unsuccessful because compound decomposed in deuteriochloroform. Anal. Calcd for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85. Found: C, 72.02; H, 7.11.

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Registry No. **1a**, 50-23-7; **1b**, 53-06-5; **1c**, 152-58-9; **1d**, 50-24-8; **1e**, 53-03-2; *cis*-**3a**, 105562-13-8; *trans*-**3a**, 105562-12-7; *cis*-**3b**, 118916-30-6; *trans*-**3b**, 118864-84-9; *cis*-**3c**, 118864-85-0; *trans*-**3c**, 118864-86-1; *cis*-**3d**, 118864-87-2; *trans*-**3b**, 118864-88-3; *cis*-**3e**, 118724-35-9; *trans*-**3e**, 118724-36-0; *cis*-**7a**, 118864-89-4; *trans*-**7a**, 118864-90-7; *cis*-**7b**, 118864-91-8; *trans*-**7b**, 118864-92-9; *cis*-**7c**, 118864-93-0; *trans*-**7c**, 118864-94-1; *cis*-**7d**, 118866-09-4; *trans*-**7d**, 118864-95-2; *cis*-**7e**, 118724-37-1; *trans*-**7e**, 118724-38-2; *cis*-**8**, 118724-39-3; *trans*-**8**, 118724-40-6; **9a**, 95811-04-4; **9b**, 95909-27-6; **10a**, 118724-41-7; **10b**, 118724-42-8; **11a**, 98039-97-5; **11b**, 98040-02-9; **12a**, 118724-43-9; **12b**, 118724-44-0; **13a**, 118724-45-1; **13b**, 118724-46-2; **14**, 118724-47-3; methyl 11β,17,20α-trihydroxy-3-oxo-1,4-pregnadien-21-oate, 97232-42-3; methyl 11β,17,20β-trihydroxy-3-oxo-1,4-pregnadien-21-oate, 97274-84-5.

A General Method for the Synthesis of Glycerophospholipids and Their Analogues via H-Phosphonate Intermediates†

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A general chemical method for the synthesis of glycerophospholipids and their analogues via H-phosphonate intermediates has been developed. It was found that 1,2-dipalmitoylglycero-3-H-phosphonate, prepared by the reaction of 1,2-dipalmitoylglycerol with PCl_3/imidazole, reacts with various hydroxylic components (choline tosylate, *N*-(*tert*-butoxycarbonyl)ethanolamine, *N*-(*tert*-butoxycarbonyl)-L-serine) in the presence of condensing agents to produce in high yield the corresponding glycero-3-H-phosphonate diesters. These can be converted into natural phospholipids via oxidation with iodine or into thio or seleno analogues by using sulfur or selenium as oxidant, respectively.

The vital role played by phospholipids in many biological processes has in the last decade stimulated a numbers of studies concerning their chemistry, biochemistry, and physical properties.[1,2] Interactions of phospholipids with biopolymers such as peptides,[3] DNA,[4] and polysaccharides of cell structures[5,6] have been extensively investigated. Phospholipid analogues were found to be a valuable tool in studies concerning elucidation of the mechanism of some enzymatic reactions,[7] in probing biomembranes structures,[8] and in the preparation of liposomes with the desired properties.[9] Also therapeutical applications of phospholipids have been investigated that use these molecules as drug carriers[10] or as drugs per se.[9,11] Such studies caused high demand for phospholipids and their analogues of unequivocal structure and have resulted in an extensive expansion in the field of chemical synthesis of phospholipids.[12,13]

The most important stage in the chemical synthesis of phospholipids is phosphorylation, which leads to formation of a phosphodiester bond, the major structural element of these compounds. Due to considerable achievements during the past years, the synthetic chemistry of phospholipids has now at its disposal a variety of phosphorylation methods which make use of phosphodiester,[12-14] phosphotriester,[13,15] and phosphite[16] chemistries. Some other methods have also been developed.[16,17]

The most straightforward one, the phosphodiester method for phospholipids synthesis, consisting of condensation of glycerol phosphate with a suitable hydroxylic component, is inefficient in terms of both yield and la-

†The H is being used to emphasize that the phosphonic acid is unsubstituted.

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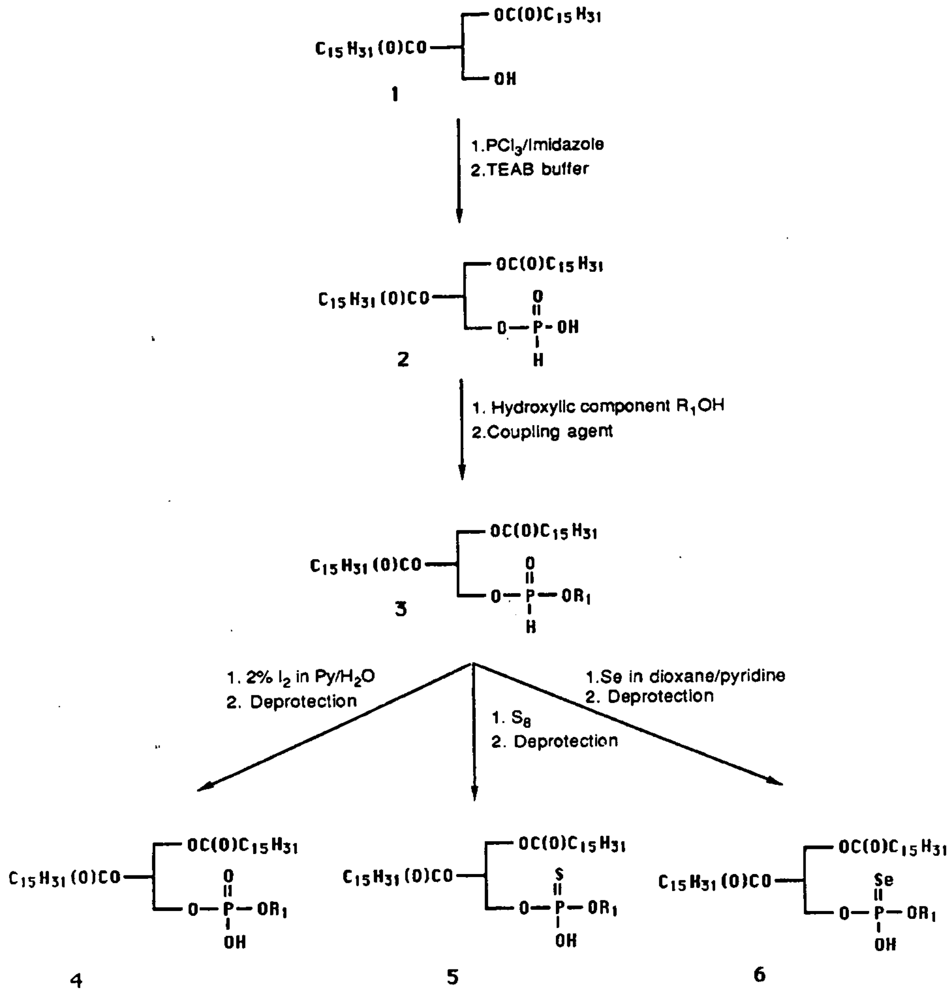
Scheme I. Synthesis of Phospholipids via H-Phosphonate Intermediates



3a, R_1= choline;
3b, R_1= N-t-butoxycarbonylethanoloamine
3c, R_1= N-t-butoxycarbonyl-L-serine

4a, R_1= choline
4b, R_1= ethanoloamine
4c, R_1= L-serine

5a,6a, R_1= N-t-butoxycarbonylethanoloamine
5b,6b, R_1= ethanoloamine
5c R_1= choline

borious experimental procedure.[14] Phosphotriester chemistry, on the other hand, usually offers higher yield during the coupling step, but this is often offset by losses of a material during the removal of phosphate protecting groups.[15] The phosphoramidite approach, especially in the recent version reported by Stec et al.[18] affords phospholipids in good yield and opens possibilities for their modification at the phosphorus center as, e.g., isotope labeling or sulfurization. Unfortunately this method relies on phosphoramidites, which are rather reactive and difficult to handle for inexperienced people, and all starting materials have to be prepared in situ.[18]

Recently, the hydrogen phosphonate method, originally designed by us for the oligonucleotide synthesis,[19,20] attracted our attention as an alternative method for a chemical synthesis of phospholipids and their conjugates. Simple experimental procedures and high yield of phosphodiester formation on one hand and easy access to phospholipid analogues on the other, could make the H-phosphonate approach the method of choice for the phospholipids synthesis.

To demonstrate the utility of H-phosphonate intermediates in the synthesis of phospholipids, 1,2-dipalmitoyl-*sn*-glycero-3-H-phosphonate (2) was prepared by the reaction of 1,2-dipalmitoyl-*sn*-glycerol (1) with the PCl_3/imidazole reagent system[21] or with salicylchlorophosphite[22] as a phosphitylating reagent (Scheme I). After purification on a silica gel column and precipitation from a hexane–ether mixture, the glycero-3-H-phosphonate 2 was obtained as a solid in over 80% yield. 1,2-Dipalmitoyl-*rac*-glycero-3-H-phosphonate was also prepared in a similar way and it was used in most studies concerning optimization.

In a typical synthesis of phospholipid diesters, the glycero-H-phosphonate 2 was rendered anhydrous by repeated evaporation of added pyridine and then condensed

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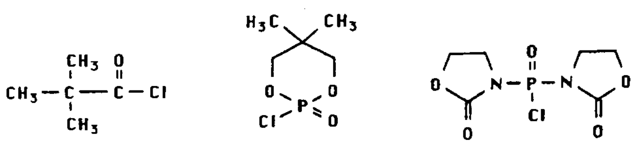
in the same solvent with a hydroxylic component, such as choline tosylate, *N*-(*tert*-butoxycarbonyl)ethanolamine, *N*-tritylethanolamine, or *N*-(*tert*-butoxycarbonyl)-L-serine in the presence of a coupling agent 7 (pivaloyl chloride, PV-Cl), 8 (5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphorinan, NPCl), or 9 (bis[2-oxo-3-oxazolidynyl]phosphinic chloride, OXP). When TLC analysis showed a complete conversion of 2 into the respective glycero-H-phosphonate diesters 3 (ca. 5 min when pivaloyl chloride was used as a condensing agent or 5–10 min in case of chlorophosphates 8 and 9), the reaction mixture was simultaneously quenched and oxidized by addition of iodine in aqueous pyridine, followed by workup and purification on a silica gel column. The total yields from 2 to phospholipids 4 were ca. 80%.

Since TLC analysis at the various stages of synthesis showed virtually quantitative conversion of substrates into the products, we ascribe the lower than expected isolated yield of phospholipids 4 due to losses of the material during silica gel chromatography rather then to incomplete coupling, oxidation, or formation of side products.

For routine synthesis of phospholipids all synthetic steps can be carried out as a "one-pot" reaction. However, if it is desired, intermediates can be isolated, purified, and then used for the subsequent transformation.

Phosphitylation of compound 1 with PCl_3/imidazole or salicylchlorophosphite proceeds fast without acyl migration (TLC, ^{31}P NMR and ^{13}C NMR analyses) and without racemization (optical rotation). For practical purpose, chromatography of the glycero-3-H-phosphonate 2 can be omitted when PCl_3/imidazole is used as a phosphitylating reagent. In case of salicylchlorophosphite, a chromatographic purification seems to be necessary in order to removed decomposition products of the reagent.

Among the condensing reagents investigated, 7–9, the most convenient one seems to be chlorophosphate 8. It is a stable, crystalline compound, easy to prepare in large quantities,[23] and has good solubility in most organic solvents. This reagent ensures clean and reasonable fast coupling (5–10 min) without danger of side reactions even if the reaction mixture is left for a longer time. The other



7 8 9

investigated chlorophosphate 9 also possesses similar properties, being an easily accessible,[24] stable, crystalline compound, and being slightly more reactive than chlorophosphate 8 in promoting condensations. The only inconvenience in using this reagent is its rather low solubility in organic solvents, which results in heterogeneous reaction mixtures, partly also because of the precipitation of decomposition products of the reagent. Pivaloyl chloride, which is commonly used in oligonucleotide synthesis,[25,26] seems to be unnecessarily reactive for the purpose of phospholipid synthesis. It may also lead to formation of side products if the reaction mixture is not quenched when the reaction is over.

To investigate the possibility of using a H-phosphonate diester of type 3 to produce phospholipids analogues having a modified phosphorus center, 1,2-dipalmitoyl-*sn*-glycero-3-H-phosphono-*N*-(*tert*-butoxycarbonyl)ethanolamine (**3b**) was obtained by using the procedure describe above and subjected to the reaction with elemental sulfur. The sulfurization was carried out under two reaction conditions, with sulfur in toluene/pyridine mixture, or by converting **3b** into the silyl ester with trimethylsilyl chloride followed by the addition of sulfur. TLC analysis showed that both methods are effective for the conversion of H-phosphonate diester **3b** into the thiophosphate derivative **5a** and that the reactions were almost complete after ca. 30 min. Removal of N-protecting groups under the standard acidic conditions[27] followed by silica gel chromatography afforded a major product in over 70% yield. However, the ^{31}P NMR spectrum of the isolated compound was not as one might expect to be for the compound **5b**, and instead of resonance(s) at ca. 60 ppm, two singlets at ca. 28 ppm were observed. The chemical shift value and the pattern of signals in the ^{31}P NMR spectrum indicated on a phosphorothioate triester containing a P(O)(SR) group and not on the desired phosphorothioate diester **5b** with a P(S)(OH) function. It was most likely that formation of a phosphorothioate triester occurred during the deprotection step. Strong acidic conditions used for the removal of the *tert*-butoxycarbonyl group from the ethanolamine moiety of **5a** may give rise to formation of *tert*-butyl carbocation, which in turn may react with the phosphorothioate diester to produce a phosphorothioate triester. To check this assumption, the ^{31}P NMR spectrum was recorded directly after sulfurization of **3b**. As expected the spectrum showed signal at ca. 60 ppm (phosphorothioate diester **5a**), and this was replaced by two singlets at ca. 28 ppm upon removal of the *tert*-butoxycarbonyl group. To eliminate this undesired reaction pathway, the deprotection was carried out in the presence of various carbocation scavengers, as e.g. anisole, thioanisole, or 1,2-ethanedithiol. As judged from TLC and from the ^{31}P NMR spectra, addition of anisole had a rather minor beneficial effect on the formation of phosphorothioate diester **5b** (two singlets at ca. 58 ppm), and thioanisole suppressed phosphorothioate triester formation by ca. 50%. The most efficient as a carbocation scavenger was found to be 1,2-ethanedithiol, which secured complete removal of *tert*-butoxycarbonyl group under acidic conditions from the ethanolamine moiety with a minimal (ca. 1–2%) formation of the phosphorothioate triesters. After such a modification of the deprotection procedure, we were able to synthesize the thio analogues **5b** and **5c** in a "one-pot" reaction in a total yield of ca. 90%.

A similar procedure was used for the preparation of seleno analogues of phospholipids. To this end, the H-phosphonate diester **3b** was produced in situ from **2**, and then a suspension of selenium in dioxane was added. The reaction was slower than that with sulfur, and the mixtures was left to stand overnight to ensure almost complete conversion to the seleno derivatives **6**. After workup and purification on a silica gel column, compounds **6a** was obtained in 98% yield. Removal of *tert*-butoxycarbonyl

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Table I. ^{13}C and ^{1}H NMR Data of Some Synthetic Phospholipids and Their Analogues[28]

compd	1-CH_2	2-CH	3-CH_2	α-CH_2O	β-CH_2	other	solvent	δ ^{31}P in ppm (solvent)
2	62.63	70.57 (7.8 Hz)	61.97 (3.7 Hz)			45.57 (CH_2-N), 8.58 (Me)	$CDCl_3$/MeOD (9:1, v/v)	4.60 (pyridine), $^1J_{PH}$ = 626 Hz, $^3J_{PH}$ = 6.8 Hz
3b	61.56	69.59 (6.4 Hz)	65.21	63.79	41.02	155.81 (C=O), 79.09 (t-Bu), 78.97 (t-Bu)	$CDCl_3$	8.65 and 8.43 (pyridine), $^1J_{PH}$ = 713 Hz, $^3J_{PH}$ = 9.3 and 9.1 Hz
	61.53	69.35 (5.5 Hz)						
4a	63.30	70.97 (8.3 Hz)	64.07 (4.6 Hz)	59.72 (4.6 Hz)	66.86	54.54 (NMe_3)	$CDCl_3$/MeOD/D_2O (50:50:15, v/v)	−0.45 (pyridine)
4b	62.76	70.61 (7.3 Hz)	69.13 (7.3 Hz)	61.96 (5.5 Hz)	40.79 (3.7 Hz)		$CDCl_3$/MeOD (4:1, v/v)	0.90 (pyridine)
4c	62.55	70.25	64.83			63.68 (CH_2, Ser), 54.11 (CH, Ser), 170.0 (C=O, Ser)	$CDCl_3$/MeOD (9:1, v/v)	0.00 (pyridine)
5b	69.13	70.74 (7.3 Hz)	64.44 (5.5 Hz) 64.35 (3.7 Hz)	62.23 (5.5 Hz)	40.59 (5.5 Hz)		$CDCl_3$/MeOD/D_2O (50:50:15, v/v)	58.44 and 58.42 (pyridine)
5c	62.90	70.24 (broad)	63.92 (5.5 Hz)	59.67	66.20 (broad)	54.76 (NMe_3)	$CDCl_3$	56.45[a,b] (pyridine)
6a	62.78	70.37 (11.0 Hz)	64.85 (7.4 Hz)	62.55 (5.5 Hz)	43.20 (3.7 Hz)	157.35 (C=O), 72.5 (t-Bu)	$CDCl_3$	52.43[a] (pyridine), $^1J_{PSe}$ = 809.8 Hz
6b	62.62	70.08 (9.1 Hz)	65.49	62.62	40.62		$CDCl_3$	55.38 and 55.23 (pyridine), $^1J_{PSe}$ = 807.4 Hz

[a] Signals from the two diastereoisomers were not resolved. [b] An enzymatic digestion with phospholipase A_2 and C[29] revealed presence of two diastereoisomers.

group from **6a** afforded **6b** in 56% yield.

All synthesized compound were characterized by TLC and spectral analysis and compared with commercial samples of natural phospholipids (**4a–c**). For the new compounds **2** and **6**, satisfactory elemental analysis data were obtained.

In conclusion, the hydrogen phosphonate approach was found to be an efficient and experimentally simple method for the phospholipid synthesis. The distinctive features of the method are (i) easy preparation of the key intermediate **2**, which can be stored for several month, (ii) coupling reactions are fast and clean, (iii) the possibility to isolate intermediates or to carry out the synthesis as a "one-pot" reaction, (iv) the lack of a protecting group at the phosphorus center simplifies the deprotection procedure, and (v) the possibility of synthesizing various phospholipid analogues, including isotope labeling, via changing the oxidation procedure.

Experimental Section

Materials and Methods. ^{13}C and ^{31}P NMR spectra were recorded on a JEOL FX-400 FT spectrometer. ^{13}C NMR spectra were referenced to the internal solvent signal, and for ^{31}P NMR spectra 1% H_3PO_4 in D_2O was used as an external standard (coaxial inner tube). TLC was carried out on Merck silica gel 60 F_{254} precoated plates with the following eluents: $CHCl_3$/MeOH/H_2O, 95:35:2 (v/v) (system A); $CHCl_3$/MeOH/H_2O, 66:33:4 (v/v) (system B); $CHCl_3$/MeOH, 1:1 (v/v) (system C); $CHCl_3$/MeOH, 5:1 (v/v) (system D); $CHCl_3$/MeOH, 6:1 (v/v) (system E); toluene/ethyl acetate, 1:1 (v/v) (system F). The spots were developed by iodine vapor or by charring after spraying with phosphomolybdic acid.

Pyridine, acetonitrile, and triethylamine were refluxed with CaH_2 overnight and then distilled and stored over molecular sieves (4 Å) or CaH_2. Tetrahydrofuran (THF) was distilled just before use from lithium aluminum hydride. Imidazole, 1,2-dipalmitoyl-*sn*-glycerol, choline chloride, and pivaloyl chloride were Aldrich commercial grade. Reference samples of **4a–c** were purchased from Sigma. *N*-(*tert*-Butoxycarbonyl)ethanolamine, *N*-(*tert*-butoxycarbonyl)-L-serine, and 1,2-dipalmitoyl-*rac*-glycerol were prepared according to the published procedures.[27] Choline tosylate was obtained from choline chloride via ion exchange.

1,2-Dipalmitoyl-*sn*-glycero-3-H-phosphonate Triethylammonium Salt (2). To a stirred solution of imidazole (1.7 g, 24.5 mmol, evaporated with toluene) in toluene (20 mL) at 0 °C was added dropwise PCl_3 (0.47 mL, 5.4 mmol) in toluene (5 mL) followed by triethylamine (1.95 mL, 14 mmol) in toluene (5 mL). Stirring was continued for 10 min, the temperature was lowered to −5 °C, and then 1,2-dipalmitoyl-*sn*-glycerol (1.0 g, 1.8 mmol, evaporated with toluene) in toluene (20 mL) was added dropwise during a period of 60 min. When TLC analysis showed a complete conversion of the starting material into a product with lower mobility, the reaction mixture was quenched by addition of water/pyridine (1:4, v/v, 100 mL). After 15 min, chloroform was added (300 mL), and the organic layer was washed with water (2 × 100 mL), dried with sodium sulfate, evaporated, and purified on a silica gel column with chloroform/methanol/water system (100:15:1, v/v). After concentration of appropriate fractions and precipitation from hexane/ethyl ether (1:1, v/v), a white powder was obtained. Yield: 1.1 g, 83%. $[\alpha]^{20}_D$ +16.8° (*c* 3.0, $CHCl_3$). Anal. Calcd for sodium salt of **2**, $C_{35}H_{68}O_7PNa$: C, 64.2; H, 10.5; P, 4.7. Found: C, 64.1; H, 10.5; P, 4.9. For the ^{13}C and ^{31}P NMR data, see Table I.

General Procedure for Synthesis of H-Phosphonate Intermediates of Type 3. Glycero-H-phosphonate **2** (0.1–0.2 mmol) and a hydroxylic component (choline tosylate, *N*-(*tert*-butoxycarbonyl)ethanolamine, or *N*-(*tert*-butoxycarbonyl)-L-serine) (1.5–2 equiv) were rendered anhydrous by evaporation of added pyridine and dissolved in the same solvent (1–2 mL), and then a condensing reagent (PV-Cl, NPCl or OXP) (2–3 equiv) was added. After the reaction was over (5–10 min, TLC), the mixture was directly subjected to further treatment (see below) in order to obtain **4**, **5**, or **6**. To isolate the intermediate **3**, the reaction mixture was quenched by addition of 0.1 M triethylammonium bicarbonate (TEAB), extracted with chloroform, and then chromatographed on a silica gel column.

General Procedure for Synthesis of Phospholipids 4. To the crude reaction mixture containing the H-phosphonate **3** was added iodine (2 equiv) in pyridine/water (98:2, v/v, 2 mL), and the mixture was stirred for 5 min. Then chloroform (30 mL) was added, the organic phase was washed with 5% aqueous sodium bisulfite, and the aqueous phase was washed back with chloroform (30 mL). The combined organic phase was concentrated to an oil under vacuum, and traces of pyridine were removed by evaporation of added toluene. If appropriate, phospholipids were subjected to deprotection by dissolving the crude reaction product in a solution containing dichloromethane (1 mL), trifluoroacetic acid (1 mL), and 70% perchloric acid (1 mL) and keeping it at 0 °C for 30 min. The reaction mixture was then diluted by addition of water (4 mL), chloroform (4 mL), and methanol (1 mL), the organic phase was washed with 0.5 M sodium carbonate (2 × 3 mL), and finally a combined aqueous phase was extracted

with chloroform (2 mL). Organic phases were combined and evaporated, and the residue was subjected to crystallization or purification on a silica gel column. Alternatively, the protected phospholipids can be purified on silica gel columns and then subjected to a deprotection step.

General Procedure for Synthesis of Phospholipid Analogues 5 and 6. To a crude reaction mixture containing the H-phosphonate 3 was added sulfur (2 equiv) in pyridine/toluene (1:1, v/v, 1 mL) or selenium (2 equiv) in dioxan (1 mL), and the suspension was stirred for 2 and 10 h, respectively. The reaction mixtures were diluted with chloroform (30 mL) and washed with water or with 0.1 TEAB. Further workup and deprotection were the same as described for 4 with the exception that the deprotection was carried out in the presence of 1,2-ethanedithiol (10 equiv).

1,2-Dipalmitoyl-*sn*-glycero-3-H-phosphono-*N*-(*tert*-butoxycarbonyl)ethanolamine (3b). Column chromatography: silica gel; eluent, toluene/ethyl acetate (1:1, v/v). Yield 98%. $[\alpha]^{20}_D$ +2.3° (*c* 2.6, CH_2CL_2). R_f 0.60 (system F). For the ^{13}C and ^{31}P NMR data, see Table I.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (4a). Column chromatography: silica gel; eluent, $CHCl_3/MeOH/H_2O$ (66:33:4, v/v). Yield 80%. $[\alpha]^{20}_D$ +7.7° (*c* 2.0, $CHCl_3$/MeOH, 1:1). R_f 0.45 (system B). For the ^{13}C and ^{31}P NMR data, see Table I.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (4b). Column chromatography: silica gel; eluent, $CHCl_3/MeOH/H_2O$ (100:15:1, v/v) and then $CHCl_3/MeOH/H_2O$ (95:35:2, v/v). Yield 86%. $[\alpha]^{20}_D$ +12.3° (*c* 2.2, $CHCl_3$/MeOH, 9:1). R_f 0.50 (system A). For the ^{13}C and ^{31}P NMR data, see Table I.

1,2-Dipalmitoyl-*sn*-glycero-3-phospho-L-serine (4c). Column chromatography: silica gel; eluent, $CHCl_3/MeOH/H_2O$ (65:25:4, v/v). Yield 81%. $[\alpha]^{20}_D$ +12.5° (*c* 1.5, $CHCl_3$/MeOH/H_2O, 65:25:4, v/v). R_f 0.40 (system B). For the ^{13}C and ^{31}P NMR data, see Table I.

1,2-Dipalmitoyl-*sn*-glycero-3-thiophosphoethanolamine (5b). Column chromatography: silica gel; eluent, $CHCl_3$/MeOH (5:1, v/v). Yield 93%. $[\alpha]^{20}_D$ +17.5° (*c* 1.3, $CHCl_3$/MeOH, 1:1, v/v). R_f 0.49 (system D). For the ^{13}C and ^{31}P NMR data, see Table I.

1,2-Dipalmitoyl-*sn*-glycero-3-thiophosphocholine (5c). Column chromatography: silica gel; eluent $CHCl_3$/MeOH (1:1, v/v). Yield 86%. $[\alpha]^{20}_D$ +16.0° (*c* 2.0, $CHCl_3$). R_f 0.60 (system C). For the ^{13}C and ^{31}P NMR data, see Table I.

1,2-Dipalmitoyl-*sn*-glycero-3-selenophospho-*N*-(*tert*-butylcarbonyl)ethanolamine (6a). Column chromatography: silica gel; eluent, $CHCl_3$/MeOH (6:1, v/v). Yield 98%. R_f 0.55 (system E). For the ^{13}C and ^{31}P NMR data, see Table I.

1,2-Dipalmitoyl-*sn*-glycero-3-selenophosphoethanolamine (6b). Column chromatography: silica gel; eluent, $CHCl_3$/MeOH (6:1, v/v). Yield 56%. $[\alpha]^{20}_D$ +17.6° (*c* 1.6, $CHCl_3$/MeOH, 2:1, v/v). R_f 0.37 (system E). For the ^{13}C and ^{31}P NMR data, see Table I. Anal. Calcd $C_{37}H_{74}O_7PNSe$: C, 58.8; H, 9.9; N, 1.9. Found: C, 58.5; H, 9.9; N, 1.7.

Acknowledgment. We are indebted to Prof. P. J. Garegg for his interest and to the Swedish National Board for Technical Development and the Swedish Natural Science Research Council for financial support.

Steady-State and Laser Flash Photolysis Studies of Norbornenobenzoquinones and Their Diels-Alder Adducts[1]

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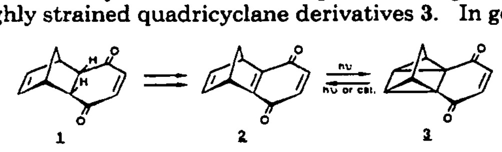
Results of a photochemical study based on nanosecond laser flash photolysis and steady-state photolysis are reported for several norbornenobenzoquinones and their rigid Diels-Alder adducts. Products isolated from steady-state photolysis of a few representative cases suggest that the preferred mode is intramolecular [2 + 2] cycloaddition wherever feasible, and triplets have been implicated in this photoreaction. The 337.1-nm laser pulse excitation of the substrates in benzene gave rise to triplets ($\lambda_{max}{}^T$ = 390-580 nm), characterized by short lifetimes (21 ns-1.05 μs) in fluid solutions at room temperature. The triplets were efficiently quenched by oxygen, ferrocene, *p*-methoxyphenol, HTEMPO, and azulene, but they exhibited reluctant quenching behavior toward DMHD and triethylamine. The lower limits of triplet yields ($\phi_{T,lim}$) for most of the substrates were measured by energy transfer to 9,10-diphenylanthracene (DPA). In some cases, the efficiency of energy transfer to DPA in benzene appeared to be small, probably owing to reversible charge transfer interaction competing favorably with energy transfer.

Introduction

The interesting tricyclic quinone 2 (2,3-norbornenobenzoquinone),[3a,b] readily available from the endo adduct 1 of 1,3-cyclopentadiene and *p*-benzoquinone, has not received much attention except for a few studies by Cookson and co-workers.[3c-e] Recently, some of us[4] have demonstrated the synthetic potential of 2 through Diels-Alder chemistry to prepare novel, strained polycyclic systems. In continuation, we became interested in utilizing the photochemistry of 2 and related quinones to gain access to highly strained quadricyclane derivatives 3. In general,



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(1) Document No. NDRL-3114 from the Notre Dame Radiation Laboratory.

(2) (a) University of Hyderabad. (b) Indian Institute of Technology, Kanpur. (c) University of Notre Dame. (d) Undergraduate research student from the University of Waterloo, Ontario, Canada. (e) Current address: 347A, Petroleum Laboratory, Research and Development, Philips Petroleum Company, Bartlesville, OK 74004.

(3) (a) Diels, O.; Alder, K. *Ber.* 1929, *62*, 2337-2372. (b) Meinwald, J.; Wiley, G. A. *J. Am. Chem. Soc.* 1958, *80*, 3667-3671. (c) Cookson, R. C.; Hill, R. R.; Hudec, J. *J. Chem. Soc.* 1964, 3043-3062. (d) Cookson, R. C.; Hill, R. R. *J. Chem. Soc.* 1963, 2023-2026. (e) Cookson, R. C.; Grundwell, E.; Hill, R. R.; Hudec, J. *J. Chem. Soc.* 1964, 3062-3075.

(4) Mehta, G.; Padma, S. *J. Am. Chem. Soc.* 1987, *109*, 7230-7232.

Table 11-1
Hydrophobic and hydrophilic units of membrane lipids

Membrane lipid	*Hydrophobic unit*	*Hydrophilic unit*
Phosphoglycerides	Fatty acid chains	Phosphorylated alcohol
Sphingomyelin	Fatty acid chain and hydrocarbon chain of sphingosine	Phosphoryl choline
Glycolipid	Fatty acid chain and hydrocarbon chain of sphingosine	One or more sugar residues
Cholesterol	Entire molecule except for OH group	OH group at C-3

MEMBRANE LIPIDS ARE AMPHIPATHIC MOLECULES CONTAINING A HYDROPHILIC AND A HYDROPHOBIC MOIETY

The repertoire of membrane lipids is extensive, perhaps even bewildering at first sight. However, they possess a critical common structural theme: *membrane lipids are amphipathic molecules* (amphiphilic molecules). They contain both a *hydrophilic* and a *hydrophobic* moiety (Table 11-1).

Let us look at a space-filling model of a phosphoglyceride, such as phosphatidyl choline (Figure 11-6). Its overall shape is roughly rectangular. The two fatty acid chains are approximately parallel to each other, whereas the phosphoryl choline moiety points in the opposite direction. Sphingomyelin has a similar conformation (Figure 11-7). The sugar group of a glycolipid occupies nearly the same position as the phosphoryl choline unit of sphingomyelin. Therefore, the following shorthand has been adopted to represent these membrane lipids. The hydrophilic unit, also called the *polar head group*, is represented by a circle, whereas the hydrocarbon tails are depicted by straight or wavy lines (Figure 11-8).

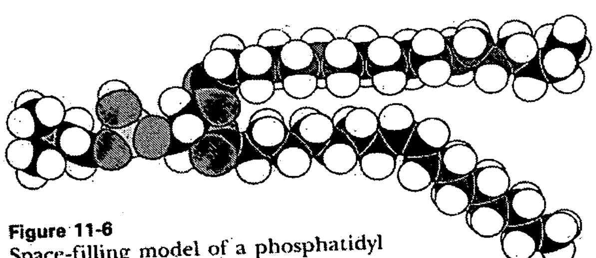

Figure 11-6
Space-filling model of a phosphatidyl choline molecule.

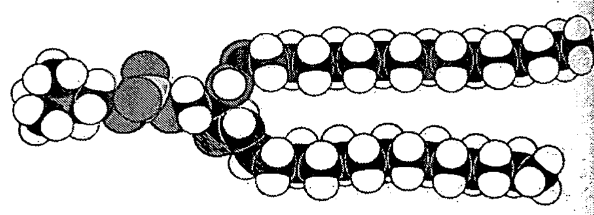

Figure 11-7
Space-filling model of a sphingomyelin molecule.

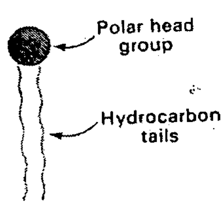

Figure 11-8
Symbol for a phospholipid or glycolipid molecule.

AMPHIPATHIC LIPIDS FORM ORIENTED MONOLAYERS AT AIR-WATER INTERFACES

In 1773, Benjamin Franklin wrote a letter to a scientist friend about the effect of oil on water. Franklin was fascinated by the ancient observation that waves in a storm can be smoothed by pouring oil into the sea. In the letter, Franklin described an experiment he carried out to learn more about this curious phenomenon. He saw one day that the water in a large pond in an English village was rough because of a strong wind, and lost no

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CHEMISTRY AND FUNCTIONAL DISTRIBUTION OF SULFOGLYCOLIPIDS

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CONTENTS

ABBREVIATIONS

A_2Gro	diacylglycerol (acyl$_2$Gro), e.g. $A_{16:0}A_{18:3}$Gro designates 1-palmitoyl-2-linolenoylglycerol
Cer	ceramide, *N*-acylsphingosine (d18:1/24h:0 designates a ceramide with sphingenine and 2-hydroxy 24:0 fatty acid)
d18:1	sphingenine (sphingosine)
d18:0	sphinganine (dihydrosphingosine)
E_2Gro	*sn*-2,3-di-*O*-alkylGro, (alkyl)$_2$Gro, when the carbon chain length is known written as follows: $E_{25}E_{20}$Gro, *sn*-2-sesterterpenyl-3-phytanylglycerol
EAGro	1-alkyl-2-acylGro, e.g. $E_{14:0}A_{16:0}$Gro designates 1-myristyl-2-palmitoylglycerol
GalSph	galactosyl sphingosine (psychosine)
h	e.g. in 24 h:0,2-hydroxy fatty acid
HSO$_3$-Chol	cholesterol 3-sulfate
PAPS	3′-phosphoadenosine 5′-phosphosulfate
SM4g	HSO$_3$-3Galβ-1-Diradylglycerols (subscript S, M, and g stands for sulfate, mono- and glycerol respectively. Diradyl means both alkylacyl and diacyl types are included), seminolipid
SM4s	GalCer I^3-sulfate, HSO$_3$-3Galβ-1Cer, galactosyl sulfatide (s stands for sphingoid)
SM4s-6	GalCer I^6-sulfate, HSO$_3$-6Galβ-1Cer
SM4s-Glc	GlcCer I^3-sulfate, HSO$_3$-3Glcβ-1Cer, glucosyl sulfatide
SM3	lactosyl sulfatide, LacCer II^3-sulfate, HSO$_3$-3Galβ-4Glcβ-1Cer.
SM2a	Gg$_3$Cer II^3-sulfate, GalNAcβ-4(HSO$_3$-3)Galβ-4Glcβ-1Cer
SM2b	Gg$_3$Cer III^3-sulfate, HSO$_3$-3GalNAcβ-4Galβ-4Glcβ-1Cer
SB1a	Gg$_4$Cer II^3,IV^3-*bis*-sulfate, HSO$_3$-3Galβ-3GalNAcβ-4(HSO$_3$-3)Galβ-4Gl cβ-1Cer
SB2	Gg$_3$Cer II^3,III^3-*bis*-sulfate, HSO$_3$-3GalNAcβ-4(HSO$_3$-3)Galβ-4Glcβ-1Ce r
SMGb$_4$	Gb$_4$Cer IV^3-sulfate, HSO$_3$-3GalNAcβ-3Galα-4Galβ-4Glcβ-1Cer
SMGb$_5$	Gb$_5$Cer V^3-sulfate, HSO$_3$-3Galβ-3GalNAcβ-3Galα-4Galβ-4Glc β-1Cer
SMGM1a	HSO$_3$-3Galβ-3GalNAcβ-4(NeuGcα2-3)Galβ -4Glcβ-1Cer
SMiGb$_4$	iGb$_4$Cer IV^3-sulfate, HSO$_3$-3GalNAcβ-3Galα-3Galβ-4Glcβ-1Cer
SMiGb$_5$	iGb$_5$Cer V^3-sulfate, HSO$_3$-3Galβ-3GalNAcβ-3Galα-3Galβ-4Glcβ-1Cer
SMUnLc$_4$Cer	SGGL-1 (U stands for uronic acid), HSO$_3$-3GlcUβ-3Galβ-4GlcNAcβ-3Galβ-4Galβ-4Glcβ-1Cer
SMUnLc$_6$Cer	(SGGL-2), HSO$_3$-3GlcUβ-3-[Galβ-4GlcNAcβ-3]$_2$Galβ-4Glcβ-1Cer
HSO$_3$-PtdGro	phosphatidylglycerosulfate
SQ-A_2Gro	6-sulfo-α-D-quinovosyldiacylglycerol
t18:0	trihydroxysphinganine (phytosphingosine)

The abbreviations and symbols for carbohydrates and lipids follow the recommendations of the IUPAC/IUBMB Commission of Biochemical Nomenclature.[213] In the present article the trivial name 'sulfatides', that has been used to designate Galβ-1Cer I^3-sulfate, is used as a generic term for a class of amphiphiles containing sulfate esters and carbo-

hydrates,[159] in the same way as 'gangliosides' include all glycolipids containing sialic acids. For other trivial names for sulfatides see Section I. B.

I. INTRODUCTION

The purpose of this chapter is to summarize the recent progress on the purification, analysis, distribution and biological function of sulfatides with reference to the sulfoamphiphiles including cholesterol 3-sulfate (HSO_3-Chol), and sulfonoglycolipids. In particular, the quantitation data have been carefully assessed and structure determination processes evaluated as critically as possible. An attempt was made to cover the literature published later than 1975 and the subjects handled in the previous reviews in this series[159,271] were described only briefly. To stay within space limitations, sometimes only reviews or the more recent works of the author were cited.

A. A Brief History

The first one of the mammalian sulfated glycolipids, galactosyl sulfatide (SM4s), was isolated by Thudichum in as early as 1884[108,159,361,639] from the human brain. However, SM4s had long been neglected as dramatically underscored by the fact that the components were identified half a century later and the correct location of the sulfate ester was not determined until 1962.[108,636]

In the next 20 years, numerous novel sulfatides were brought to light,[361] uncovering a distinct group of acidic amphiphiles comparable to gangliosides. The variety of sulfatides discovered at that time included 2,3,6,6′-tetraacyl-trehalose-2′-sulfate from Mycobacteria,[149] HSO_3-3Galpβ-6Manpα-2Glcpα-1$E_{20}E_{20}$Gro from an extremely halophilic archaea,[273,275] lactosyl sulfatide (SM3) from human kidney,[366] seminolipid (SM4g) from mammalian testis,[232,238] ganglioside sulfates from echinoderms,[297,457] and the ganglio- and globo-series sulfatides from mammalian kidneys.[400,561] Most of these compounds were discovered by metabolic labeling with [^{35}S]sulfate.[159,238] The structure determination has greatly accelerated by the remarkable advances in MS as well as ^{1}H- and ^{13}C-NMR. Figure 1 shows some representative sulfatides and a sulfonolipid with their major lipophilic species.

Since the 1970s, metabolism of SM4s in the myelin of the central nervous system of normal and metachromatic leukodystrophy (MLD) patients was extensively studied.[108] It had been assumed, however, that sulfatides might be functioning as one of the membrane matrices. The breakthrough was brought about by the recognition that SM4s was enriched in glandular epithelial tissues of mammals,[267,361] and increased in the organs related to sodium excretion following NaCl loading to animals.[270] This increase occurred at an individual cellular level[426] by the upregulation of GalCer sulfotransferase.[231] Further, the metabolic activities of animals and the concentration of renal sulfatide sulfate could be stoichiometrically correlated by using the allometric equation.[398] To elucidate the diversity of anionic glycolipids in the biosphere, two major principles of genetics and biology, the neutral theory of molecular evolution,[238] and the allometric law[398] respectively, were introduced. In fact, the anionic groups of amphiphiles including phosphates, phosphonates, sialic acids, and sulfates, were interchangeable in many tissues and cells.[212,238] Recently, the discovery of several examples of horizontal descendence of genes related to anionic glycolipids,[501] the upregulation of GlcCer in the myelin of galactosyltransferase-deficient mice[75] as well as the studies on sulfonoglycolipid-deficient strains of photosynthetic microorganisms[155] supported the above hypotheses. Between 1944 and 1956 the role of adenosine 3′-phosphate 5′-sulfophosphate (PAPS) was elucidated by Lipmann's group and others, although the purification of the GalCer sulfotransferase to homogeneity[211] and cloning of the cDNA had to wait until 1997.[210] The cDNAs of cerebroside sulfatase and saposins were already cloned in the early 1990s.

The specific interaction of sulfatides with various mammalian proteins has stimulated studies on the possible ligand and receptor activities of sulfated amphiphiles.[107]

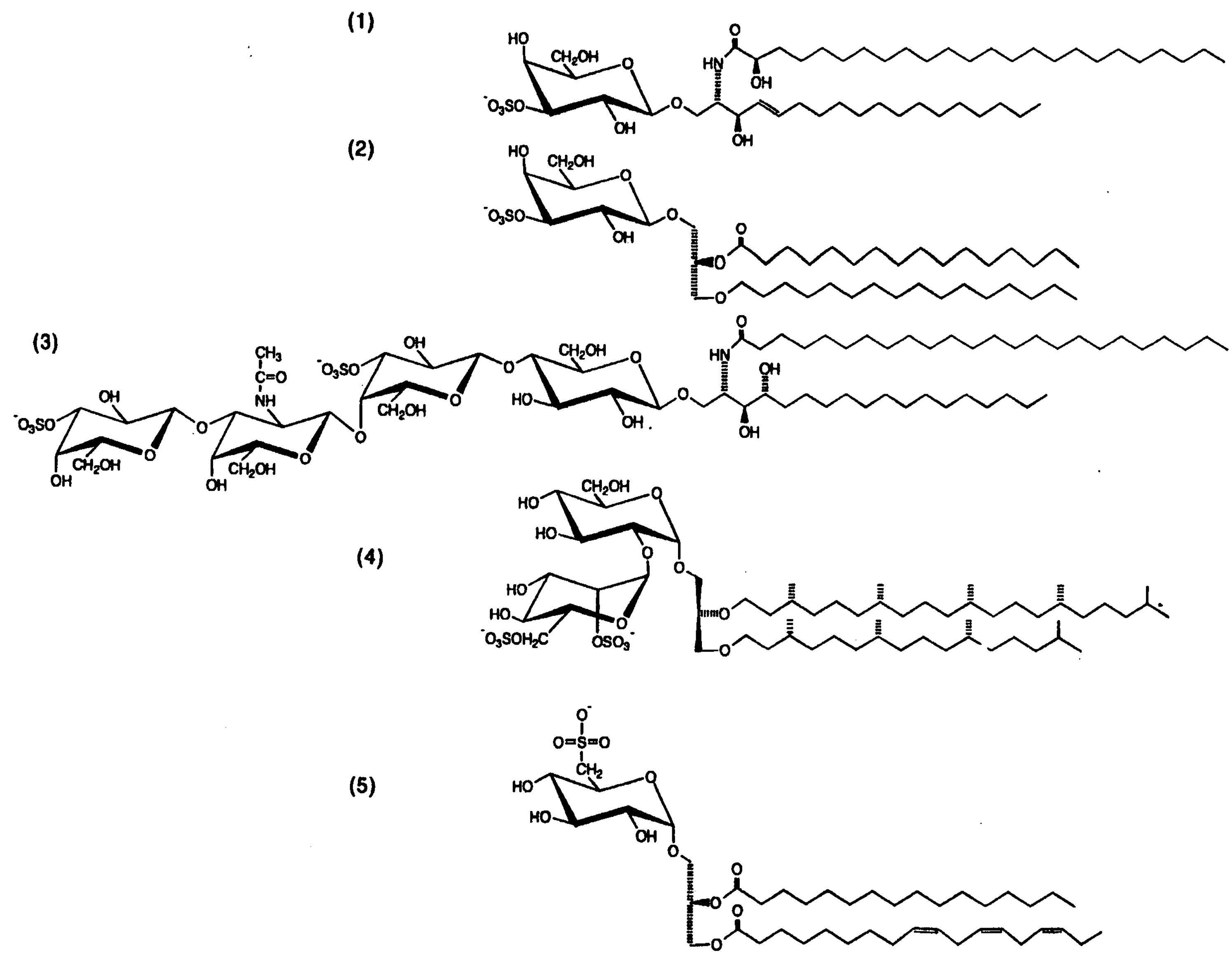


Fig. 1. The structure of representative sulfatides and a sulfonoglycolipid. (1) SM4s (d18:1/24h:0), sulfatide; (2) SM4g ($E_{16:0}A_{16:0}$Gro), seminolipid; (3) SB1a (t18:0/24:0), *bis*-sulfo-gangliotetraosylceramide; (4) *bis*-sulfo-Manα-2Glcα-3$E_{25}E_{20}$Gro; (5) SQ-$A_{18:3}$ $A_{16:0}$Gro (16:0/18:3), 1,2-diacyl-3-*O*-(6′-sulphono-α-D-quinovopyranosyl)-*sn*-glycerol.

Sulfatides interacted specifically with cell adhesive proteins including thrombospondins, and laminins,[468] with vascular system proteins including selectins,[613] as well as with pathogenic viri and mycoplasmas.[314,470] Sulfatides are increased in some human cancers originated from glandular epithelia including pulmonary cells, hepatocytes and renal tubular cells. Aberrant expression of some minor sulfatides in cancer tissues was tested for capability as tumor markers.[136,205] The discovery of these biological characteristics of sulfatides increased publications on sulfatides sharply in the 1990s.

B. Classification and Nomenclature

Glycolipids can be classified into neutral and acidic groups.[213] Acidic glycolipids contain one or more anionic residues, including acidic carbohydrates (e.g. uronic acid, and sialic acid), esters of phosphate or sulfate (R-OSO_3H where R is the neutral glycolipid), C-P (phosphonate), and C-S (sulfonate, R-SO_3H) compounds.[159,537] In addition to the ionic sulfate monoesters (half-esters of sulfuric acid), nonionic diesters (R-OSO_2*O*-sugar) and cyclic sulfates[603] have been chemically synthesized but never isolated from biological sources.[159] Although sulfatides with two sulfate groups have sometimes been designated as disulfate,[537] '*bis*-sulfate' as in Gg_3Cer II^3,III^3-*bis*-sulfate,[558] and Manα-2Glcα-3E_2Gro $II^{2,6}$-*bis*-sulfate[369] may be more appropriate. Sulfoamphiphiles can also be classified into sulfoglycoglycerolipids,[238,273] sulfoglycosphingolipids,[160,361,505,554] and steroid sulfates[105] according to their aglycons. Because the terms sulfatide, sulfatides, sulphatide(s), and sulfoglycolipid(s) are synonyms but recognized as the different words in the databases including Medline, the papers listed will be doubled by 'or-search' on the abstracts including all of these words in addition to the keyword 'sulfatides'. The Complex Carbohydrate Structure Database (CCSD) version 17 (1997), containing more than 48,000 records on glycoconjugate structures including sulfoglycoconjugates, is accompanied by the 'CarbBank' software operated in the Windows 95 system. The database and software can be downloaded through the WorldWide Web, www.ncbi.nlm.nih.gov/. LipidBank is a database which contains records on sulfoglycolipids [information available from Dr M. Oshima (E-mail: oshima@imcj.go.jp)] and LIPIDAT database contains informations concerning synthetic and biologically derived polar lipid phase behavior.[310]

Some examples of trivial names and symbols for sulfatides currently under use include S-GalCer (sulfo-galactosylceramide);[555,657] CSE (cerebroside sulfuric ester);[159] CBS (cerebroside sulfate),[534] SGC (sulfogalactosylceramide), sulfoGalCer,[395] and SUL[27] for SM4s;[233] seminolipid,[232] SGDG (sulfogalactosyldiglyceride), SGG (sulfogalactosyl glycerolipid),[343] SGalAAG,[135,238] and SulfoGalAAG[395] for SM4g;[233] S-LacCer for SM3;[88,555] sulfoglucuronyl glycolipids (SGGL) for SMGlcUnLc$_4$Cer and SMGlcUnLc$_6$Cer; S_{tri1}, and S_{tri2} for SM2a and SB2.[246]

II. ISOLATION AND PURIFICATION

Isolation and purification of individual sulfatides are prerequisite for structural determination and quantitative analysis. It is desirable that the tissue concentration values are routinely corrected for recoveries.

A. Extraction

The media most frequently used for the extraction of total lipids containing sulfatides are chloroform/methanol/water mixtures.[320] Folch–Suzuki procedure is the method of choice for relatively polar amphiphiles including gangliosides and sulfatides.[50,116,160,480,505,547] In order to extract acidic glycolipids quantitatively, univalent cations must be present. These are usually supplied in adequate amounts by the tissue itself, but for the second and third extraction, an addition of Na^+ or K^+ is rec-

ommended,[532] while an excess of salt in the extraction medium will result in the precipitation of SM4s by the effect of salting out.[180]

1. Folch–Suzuki Method

The standard extraction procedure for mammalian sulfoglycosphingolipids is as follows. Typically, rat kidneys (50 g) were extracted in three steps with: (1) 19 vol of chloroform/methanol (2:1, v/v); (2) 10 vol of chloroform/methanol/0.88% KCl (60:120:9, v/v); and (3) 10 vol of 40 mM sodium acetate in chloroform/methanol/water (30:60:8).[560] The combined extracts were concentrated to dryness and treated with 20 ml of 0.2 M NaOH in methanol at 37°C for 1 hr to degrade acyl ester lipids. After adjustment of pH to 7.0 with 0.2 M HCl in methanol, the reaction mixture was partitioned in the Folch system. The clear upper phase was concentrated to approx. 1/10 vol, dialyzed, and lyophilized. The lyophilized extract was combined with the lower phase, made up to 500 ml of chloroform/methanol/water (5:10:1, v/v) by an addition of solvents, and applied to a column of DEAE–Sephadex.

Highly polar lipids (polysialosyl gangliosides, the ganglio-series sulfatides,[563] SMUnLc$_4$Cer and SMUnLc$_6$Cer),[68] inorganic salts and nonlipids move into the upper aqueous phase (chloroform/methanol/KCl, 3:48:47) and less polar lipids (HSO$_3$-Chol, SM4g, SM4s, Gb$_4$Cer and GM4) can be recovered in the lower organic phase (chloroform/methanol/KCl, 84:14:1) of the Folch partition system. When a salt is not included in the aqueous phase substantial portions of HSO$_3$-Chol[422] and SM4s[228] will be lost in the upper aqueous phase or in the 'fluff' which forms at the interface between the water and chloroform.[388] In the presence of 0.1% KCl, for instance, 85% of dehydroepiandrosterone sulfate was found in the upper phase, while 90% of HSO$_3$-Chol was recovered in the lower phase.[388] About 5% of the weight of the lower phase was KCl when this salt was used to wash extracts of brain tissues.[480]

It has been reported that several washings with the simulated (theoretical) upper phase (chloroform/methanol/water, 3:48:47, or more simply methanol/water, 1:1) were necessary to remove inorganic sulfate from the extract containing SM4s. In our experience, 3 washings were sufficient to remove inorganic [^{35}S]sulfate by Folch's partition[228,555] using the upper phases containing 0.88% KCl.[480] The final lower phase, after 3 washings, contained $97.3 \pm 0.5\%$ of SM4s before washing, while when no salts were included in the upper phase, only $10.9 \pm 0.1\%$ was recovered in the lower phase after 3 washings.[228,563] Lyso-SM4s,[569] lyso-SM4g[606] and lyso-SQ-A$_2$Gro[118] were distributed almost quantitatively in the aqueous phase.

2. Bligh–Dyer Method

A method for extraction of lipids using a smaller amount of solvents was developed for economical and practical reasons. The Bligh–Dyer extraction method has often been applied to bacterial suspensions that contain a large amount of water[272,273,498] or sometimes used for large-scale extraction of animal tissues. Typically, the suspensions were extracted with chloroform/methanol/water, 1:2:0.8 (v/v), then each vol of chloroform and water was added. The mixture was cleared by centrifugation, to separate upper aqueous and lower organic phases in a volume ratio of 0.9:1 with protein precipitates in the middle.[332] This extraction method may be unsatisfactory for plant lipid[63] and a larger amount of solvent may result in better recoveries.[419] In our experience, only 50% of human kidney SM3 was procured by Bligh–Dyer extraction.

3. 2-Propanol/Hexane/Water System

Halogenated hydrocarbon solvents, including chloroform, are ecologically hazardous and have strong absorption in the range below 245 nm used for U.V. detection of lipids after HPLC separation.[194] A hexane/2-propanol mixture has been used for a large-scale

extraction of phospholipids and glycolipids from brain tissue.[180] After treatment with NaOH, nonpolar lipids were effectively removed into the upper organic layer leaving a glycolipid fraction (containing 96% of SM4s) practically free of phospholipids. The periodic acid oxidation step of this procedure could be omitted.[386] Lipids were also extracted from extraneural tissues such as erythrocyte membranes,[160] liver,[204, 378] and hybridoma cells[194] with 2-propanol/ hexane/water mixture or ethylacetate/methanol/water.[194]

B. *Desalting and Salt Forms*

1. *Removal of Salts and Hydrophilic Contaminants*

Dialysis through nitrocellulose membranes (e.g. Spectrapor™ dialysis tube with the pore size of Mw 3000) against water is the simplest and, in most cases satisfactory, method to remove not only salts but also other low molecular hydrophilic compounds such as oligosaccharides, nucleotide sugars or oligopeptides[32, 50] from a suspension of sulfatides in water. Prolonged dialysis (> 48 hr) is usually avoided to minimize the loss of the monomers of gangliosides and SM4s[47, 160] present in amounts below the critical micellar concentrations (CMC, approx. 150 μg/ml for gangliosides) through nitrocellulose tubings. Because the CMC of SM4s/SM4g and lyso-SM4s/lyso-SM4g is reported to be > 3 μM[30, 455] or < 10–40 μM and < 100 μM respectively[115] and the size of micelles is about 10^6 Da,[179] being larger than those of gangliosides,[404] the loss by dialysis may occur more slowly.

Removal of water-soluble contaminants from HSO_3-Chol, SM4s, and SM4g[228] is easily achieved by Folch partition with high recoveries,[563] although this procedure is not applicable to more hydrophilic amphiphiles such as lyso-SM4s (HSO_3-3GalSph),[109] SM3, SM2a and SB1a.[563] Partition chromatographies in chloroform/methanol/water, 120:60:9,[625] or chloroform[63] using Sephadex G-25 (superfine),[109, 332, 480, 479, 555] or Sephadex LH-20 column[219] have been used to remove [^{35}S]PAPS from SM4s[356] and SM3[555] with satisfactory recoveries. Sephadex G-25 separated some higher acidic lipids including SMUnLc$_4$Cer probably due to their negative charges.[221] For a smaller scale, reversed phase chromatography using Sep-Pak C_{18},[244, 594] BondElut[262, 505, 536] or silica gel RP-18[244] was also able to remove the bulk of salts or nucleotides from SM4s,[51, 137] lyso-SM4s,[570] SM2a,[402] permethylated uronic methyl esters of SMUnLc$_4$Cer,[336] SMUnLc$_4$Cer,[163] SMUnLc$_6$Cer,[381] gangliosides,[49] and the synthetic neoglycolipids coupled with phosphatidylethanolamine.[112] The recovery was approx. 79% for SM2a.[402] Salts were successfully removed from an HSO_3-Chol fraction by a silicic acid column.[39]

2. *Salt-Forms*

As the pKa of the sulfate half-ester, e.g. in sucrose polysulfate, is from 0.43 to 1.19,[590] the salts of SM4s are fully ionized in solution above pH 3.5 (M^+ SO_3^--*O*-3GalCer) and are more strongly acidic than phosphomonoesters, carboxyl groups of monosialosyl gangliosides or phosphatidylserine[458] (cf. Figs 2 and 3). A concentrated solution of free sulfatide is strongly acidic and leads to cleavage of the ester bond. To avoid desulfation, free sulfatides should be neutralized by NH_4OH or $NaHCO_3$ and stored in the form of salts. The potassium or lithium salts have been recommended for the sake of stability.[159] When stored in water at 4°C,[32] even the salts of SM4s gradually lost sulfate over several months. Dilute solutions of the K-salt of SM4s in the solvent mixture of Dabrowski (deuterated dimethylsulfoxide/deuterated water, 98:2) also released a few percent of sulfate ester at ambient temperature over several years.

The ammonium salts of sulfatides, eluted from a DEAE–Sephadex column with solvents containing ammonium acetate, were stable for years in a dessicator.[478] Exceptionally, the ammonium salt of tetraacylated trehalose 2-sulfates obtained by elution with chloroform–methanol containing NH_4OH or ammonium acetate was unstable under most conditions of storage.[149] The spontaneous desulfation in this case was

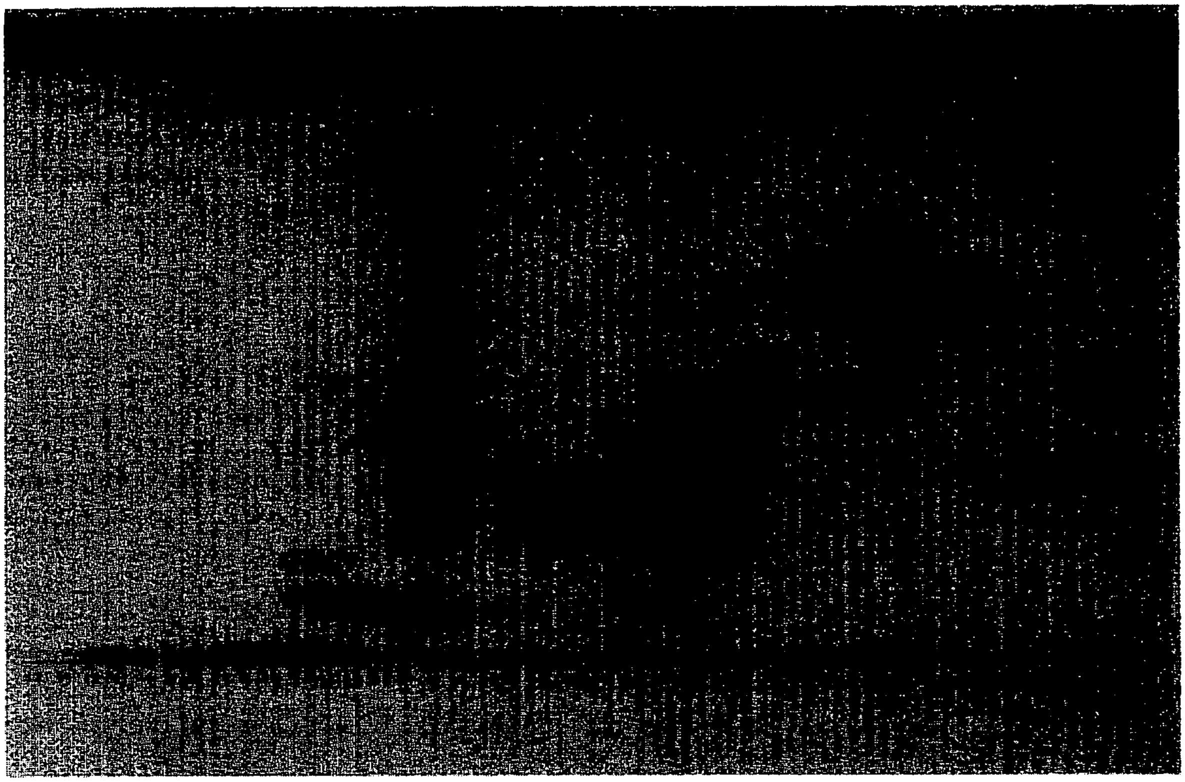


Fig. 2. DEAE–Sephadex column chromatography of acidic lipids from boar testis. Acidic lipids were eluted with a gradient of ammonium acetate in chloroform/methanol/water, 5:10:1. Fractions were separated on a HPTLC plate in chloroform/methanol/acetone/acetic acid/water, 8:2:4:2:1, and visualized with orcinol/sulfuric acid. PI, phosphatidylinositol; PS, phosphatidylserine.

assumed to be autocatalytic owing to the sulfate anion produced,[159] and it was essentially inhibited by water. To avoid spontaneous solvolysis, Goren stored sulfatides as solutions in hexane, kept over a small amount of aqueous $NaHCO_3$ to suppress the hydrolysis and to continuously neutralize any traces of acid that were formed.

To convert the mixed cationic salt into K salt, less polar sulfolipids (HSO_3-Chol, SM4s, SM4g) are simply partitioned in a Folch system containing 0.88% KCl.[232] For more complete exchange, sulfolipids were once acidified by dissolution in chloroform/methanol/0.1–0.2 M HCl.[275,327,602] The organic phase, containing the free acid form of sulfolipids, was immediately neutralized (to pH 8–9) by the addition of NH_4OH, pyridinium chloride,[94] NaOH, or KOH to final concentrations of 0.01–0.2 M to yield the desired salt form of the sulfolipid and was then freed from unbound cations by a desalting procedure. Alternatively, glycolipid sulfates were dissolved in chloroform/methanol/water, 1:1:0.05 and converted to their free acid forms by passing the solution through a small column of cation exchanger (H^+ form).[383] The free acid in the combined eluates was converted to the ammonium salt by the addition of methanolic NH_4OH. The affinity of the sulfate group in SM4s for cations is similar to that of sialic acids in gangliosides[404] and in the order of $Ca^{2+} > Mg^{2+} > K^+ > Na^+ > Li^+$.[7]

C. Chromatographic Procedures

Generally, column chromatographic separation of sulfatides is achieved by a combination of ion-exchangers and silica beads,[228,365,480,561] while for a relatively simple lipid mixture, such as the total lipid from halophilic bacteria, a silicic acid column only may be sufficient.[332,369] Elimination of gangliosides by treatment with sialidase may reduce the loading of lipids and improve resolution of SM2a,[233,559] and SMUnLc$_4$Cer.[68,221] Convenient synopses are available on the substrates, solvents for chromatographic separation and the retention volumes or Rf values of glycoglycerolipids,[116] and glycosphingolipids.[547]

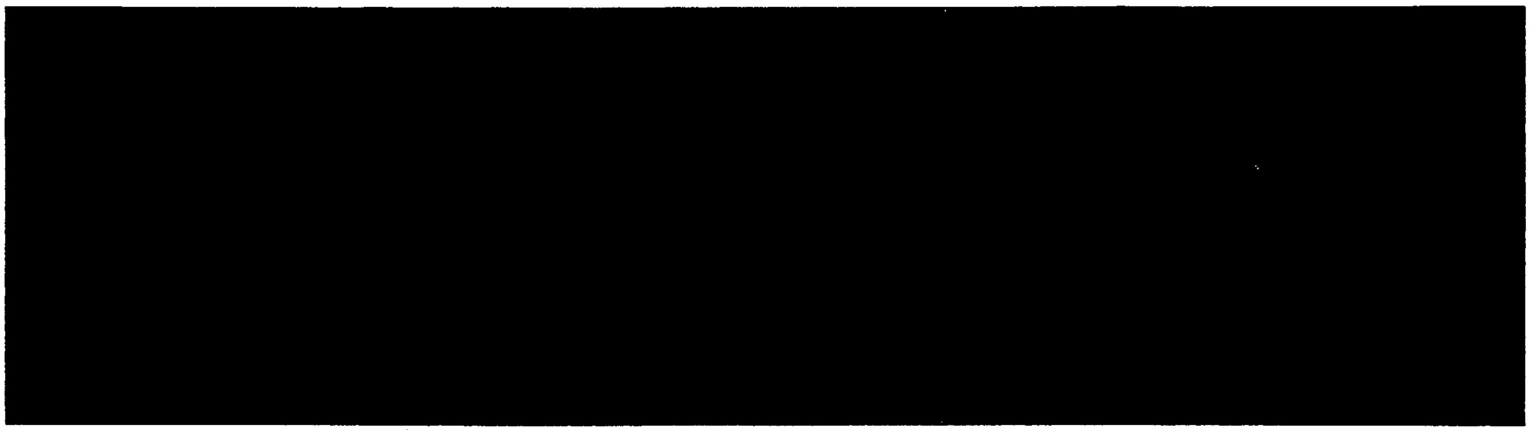


Fig. 3. Separation of acidic sphingoglycolipids from rat kidney by column chromatography using DEAE–Sephadex. Acidic lipids were eluted with a gradient of ammonium acetate in chloroform/methanol/water, 5:10:1. Each 1/2000 vol of the eluate of tube 1–120 and 1/200 vol of tube 121–288 were separated on HPTLC plates (5 × 10 cm, Merck) in chloroform/methanol/0.2% $CaCl_2$, 60:40:9 and stained with orcinol/sulfuric acid. BA, acidic glycolipid mixture from rat brain, containing a band under the double band of SM4s, is lyso-SM4g derived from SM4g by mild alkali treatment. The lowest four bands are gangliosides GM1, GD1a, GD1b, and GT1b.

1. Thin-layer Chromatography

Silica gel high-performance TLC (HPTLC) is indispensable for the studies of anionic amphiphiles[391] whereas cellulose TLC[332] is used less often for intact sulfatides.

(a) Adsorbents and solvent systems

Prewashing of the plate with methanol and activation of silica gel by heating at 100°C for 10 min or storing in a dessicator may eliminate soluble contaminants of silica gel and improve the resolution.[644] With total or alkali-resistant lipid mixtures containing a substantial amount of cholesterol or detergents, double development of the plate, first with a nonpolar solvent system (e.g. chloroform/methanol/water, 90:12:1) to remove nonpolar contaminants to the solvent front[400,494,555] and then with the second solvent system to separate the desired sulfatides, may improve the resolution greatly. Equilibration of tank vapor using a small motor-driven propeller accelerated saturation with the solvent system.[244]

In *neutral* solvent systems (Fig. 3) (e.g. chloroform/methanol/water, 65:25:4,[130,232,342,479] SM4g can be separated from SM4s, although SM4s, lyso-SM4g, and SM2a migrate close to HSO_3-Chol,[583] SM3, and ganglioside GM4 respectively. In chloroform/methanol/water (0.2% $CaCl_2$, 60:30:6,[563] SM2a migrates a little slower than the neutral analogue, Gg_3Cer; while the *bis*-sulfo-derivative, SB2, migrates much slower than its monosulfo-counterpart SM2a.[560] SM1a, the sulfate analogue of GM1a, is unique in migrating a little faster than Gg_4Cer.[561] Higher monosulfoglycolipids, $SMiGb_4Cer$ and $SMiGb_5Cer$, comigrate with *bis*-sulfoglycolipids SB2, and SB1a respectively. SM4s, SM3, SM2a, and SB1a migrate much faster than the corresponding sialic acid derivatives, GM4, GM3, GM2 and GD1a. In chloroform/methanol/water solvent systems, lyso-SM4s migrates the distance corresponding to about 2/3 of SM4s,[307,432] which is larger than those of GalSph (galactosylpsychosine) and GlcSph by the effect of the $-NH_2$ group. In contrast, lyso-SM4s comigrates with GalSph behaving like the sphingosylphosphocholine-HCl salt with the acidic and alkaline media.[569] In *basic* solvent systems, e.g. chloroform/methanol/3.5 M NH_4OH, 60:40:9,[568] SM4s comigrates with GalCer. Also HSO_3-Chol, and SM4s with nonhydroxy fatty acids are not separated from SM4s-Glc (t18:0). In 1-propanol/15 N NH_4OH/H_2O, 80:5:15,[323,425,433] bovine brain SM4s is separated into four bands on top of GalCer, because sulfatides migrate faster when ammonium ion concentration is higher.[634]

HSO_3-Chol,[384,480] SM4g,[119,228] SM4s with nonhydroxy fatty acids,[17,51,234,314] SM4s-6,[232] SM4s-Glc with t18:0,[217] lyso-SM4g as well as mono- and *bis*-sulfo-Gg_3Cer[558] can be neatly separated in *acidic* solvent systems (Fig. 2) including chloroform/methanol/acetone/acetic acid/water, 10:2:4:2:1. More polar acidic solvent systems may be suitable for more complex sulfo-glycolipids, including SM3 to SB1a,[400,558] and mono- and *bis*-sulfated disaccharide E_2Gro.[369] As in the neutral solvent systems, however, lyso-SM4g and SM4s with hydroxy fatty acids comigrate with SM3 and HSO_3-Chol respectively. Butanol/acetic acid/water, 6:2:2 has been successfully applied to SM4s and lyso-SM4s.[569]

Total lipid extracts from various sources have been separated by *two-dimensional*(2D) TLC[116,237,480] using a couple of solvent systems selected from the basic, acidic or neutral solvent systems.[237,466,547,574,606] Rat kidney acidic lipids were separated on a NH_2-silica gel HPTLC plate developed with chloroform/methanol/1% diethylamine, 50:47:15[623] for the first-dimension. The bands were then transferred to an ordinary silica gel plate and developed to the second dimension in a neutral solvent system to separate mono- (SM4s, SM3), and *bis*-sulfoglycolipids (SB2, SB1a) from mono- (GM3, GM4) and disialosyl gangliosides (GD3) respectively. Alternatively, Analtech diphasic plate with an NH_2-modified silica gel lower hemisphere resulted in excellent separation of SM4g in sperm lipids.[10]

Extraction of sulfatides from silica gel is susceptible to low recovery (about 92% for HSO_3-Chol, 70% for SM4s[282] or SM4g, and much less for higher sulfatides). Usually the band of sulfatides is moistened with water before scraping to deactivate the adsor-

bent and individual glycolipid bands were eluted from the silica scrapings by using chloroform/methanol/water mixture (e.g. 10:10:1).[383,412,539] To increase the recovery, the triglycosyl glycolipid sulfate was eluted from TLC silica gel with chloroform/methanol/0.1 N HCl, 1:2:0.8.[275] The overall recoveries of SM4g from chicken retina,[95] rat brain,[228] and human testis[606] through purification procedures using Folch extraction, silicic acid or DEAE-cellulose columns, and preparative TLC were between 80 and 90% as assessed from the recoveries of [^{35}S]-labeled sulfatides or lipid galactose. Without an appropriate cleanup procedure, silicic acid and binder may contaminate in the extract from silica gel resulting in mass spectra of high noise levels.

Glycolipids and phospholipids can be efficiently transferred from an HPTLC plate to a polyvinylidene difluoride (PVDF) membrane by a simple blotting procedure.[574] The developed HPTLC plate is dipped in a solvent mixture (2-propanol/0.2% $CaCl_2$/methanol, 40/20/7 v/v) for blotting, after which first a PVDF membrane, then a glass microfiber filter are placed on the plate and the assemblage pressed for 30 s by heating with a 180°C iron. The efficiency of transfer for each 5 μg of glycolipids was 68–93% (average 82%), and approx. 77% for SM4s.[574] The glycolipids on the membrane can be detected chemically, or immunologically, and reextracted or analyzed by LSIMS.[573]

(b) Visualization

(i) Chemical methods. The cationic dye Azure A can stain sulfo-[217,536] and sulfono-conjugates[321] with satisfactory specificity. TLC plates are either dipped in or sprayed with a saturated solution of Azure A in 1 mM H_2SO_4 (2 g in 100 ml).[217,504] The plates are washed by soaking in a mixture of methanol/40 mM H_2SO_4, 1:3, occasionally with gentle agitation and a few times of the change of the solution until the background staining is minimal. An excess of methanol in the washing solution may lead to the loss of nonpolar sulfatides such as SM4g, and an excess of water may destroy the layer of adsorbents. Aluminum-backed plates[112,125,221,295,399] (e.g. Merck) are better than glass or plastic plates (e.g. the products of Marchery-Nagel, Brinkman[27] and Baker's[204]) and have the best strength with concomitant reduction in the resolution.[549] As little as 250 pmol of SM4s is apparent to the naked eye and can be assayed by densitometry.[369,387] The color may be retained for years at ambient temperature and without cover. Sulfatides with a sulfate ester on the internal galactose (SM2a, SM1a) are stained much less intensely than sulfatides with a sulfate ester on the terminal galactose (SM4s, SM3, and SM1b). Sialic acid-containing glycolipids and acidic phospholipids are stained weakly although the bands gradually change to brown in a few days unless the plate is covered with a glass plate or Saran™ (vinylidene polymer plastics) wraps. Also a dilute solution of toluidine blue stained SM4s on cellulose paper[162] and methylene blue can detect sterol sulfates on TLC plates.[40]

Exposure of the plate to iodine vapor,[116,365,466,547] or immersing in a solution of Coomasie brilliant blue[408] stain HSO_3-Chol and sulfatides nonspecifically. Observation under U.V. light after spraying rhodamine[167,288] or primulin[112,573] visualize all species of lipids. Primulin spray and observation under U.V. light did not interfere with mass spectrometry.[574] When the saccharide is sulfated at position 2, or 3 of hexose or hexosamine, staining by periodate-Schiff reagent for detection of 1,2-glycol may be negative.[116] Sulfated hexoses, 6-sulfoquinovose, and HSO_3-Chol, produce purple, greenish, and bluish purple color by anthrone sulfuric acid, Molisch[647] or orcinol sulfuric acid stain[217] respectively.

(ii) Densitometric and chemical analysis of sulfatides. Azure A staining specific to sulfate ester has been successfully applied to quantitative assay of 0.5–4.0 nmol of sulfo-glycolipids (e.g. SM4s, *bis*-sulfo-ManGlc-E_2Gro)[217,369] on TLC plate. It should be noted that every densitometric assay needs an appropriate standard compound because the molar absorption depends on the structure of sulfatides. Nonspecific staining with cupric acetate in aqueous phosphoric acid[49,130,569,644] or

Coomasie blue and densitometry are able to quantitate 0.5–10 nmol of SM4s[408] above 0.5–1.0 nmol of SM4s,[504] and SM4g,[11] and HSO_3-Chol.[384] Sulfatides were assayed densitometrically after staining with orcinol-H_2SO_4 reagent[318,412,423,453] when hexose composition is known. Alternatively, the band of glycolipid on silica gel powder scraped from the plate was hydrolyzed without extraction[420] and component galactose analyzed enzymatically using galactose dehydrogenase and a fluorometer.[97,228,606] HSO_3-Chol was able to be determined by the sophisticated gas chromatographic method after solvolysis using 5-α-cholestane,[243,427] [4- ^{14}C] HSO_3-Chol,[348] or β-sitosteryl sulfate[40] as internal standards.

(iii) *Radioactivities.* [^{35}S]-labeled sulfo- and sulfono-glycolipids (cf. VI. A. 1) were detected by radioscan of TLC plates, autoradiography using X-ray films,[111,234,632] or by using imaging plates. Cold SM4s can be ^{3}H-labeled by tritiating with $^3H_2$ at the C4 and C5 positions of their Cer moiety.[616] High resolution is guaranteed for autoradiography while the sensitivity is not satisfactory.[466] Detection of a doublet band of SM4s containing 200–300 dpm of [^{35}S]sulfate takes more than 1 month, although the β-emission energy of [^{35}S] is approx. 10-fold of ^{3}H and comparable to ^{14}C. Use of *En^{3}Hance*™ and storage in a deep freezer at −80°C greatly improve the sensitivity of ^{3}H-labeled compounds[173] but not substantially contribute to the intensity of [^{35}S]-bands. The densitometric quantitation of autoradiography film was linear between 170 and 300 dpm of [^{35}S]-SM4s and proportional to the counts obtained by the low-level liquid scintillation assay (Low-Level™, Packard) of the band of silica gel.[443]

Conventionally, the silica gel powder scraped from TLC plates was either directly counted with the scintillation solution (e.g. Aquasol),[234] or sulfolipids extracted from the gel were assayed for radioactivities.[632,634] The limit of detection depended on specific radioactivities and detection methods. The detection/quantification imaging system BAS 2000 or 1500 (Fuji Film), with resolution higher than β-cameras and sensitivity higher than X-ray films, is rapidly replacing autoradiography and liquid scintillation counting.[254,425] The exposure time of radioluminography has been shortened to 1/10 of the time required for autoradiography with [^{35}S]-

Table 1. Antibodies interacting with sulfatides

Polyclonal
Sera from spontaneously diabetic BB rats, (+) SM4s (38%); sera from insulin-dependent diabetes patients, (+) SM4s (88%)[56,122]
Rat sera (Heyman's nephritis) or the serum of rabbits immunized with rat proximal tubule, TLC-OL, ELISA, (+) SM2a, SB2[246]
Rabbit serum, antigen: SM4s, (+) SM4s; (−) GalCer, Gal-A_2Gro[521]. Rabbit serum, SM4s, (+) SM4s, immunohistochemistry, (+) the initial part of the distal tubules or the thick ascending limb of rat kidney; (−) glomeruli, proximal tubules.[649] Rabbit sera (rSulf-1 and rSulf-2), SM4s, (+) SM4s.[462] Rabbit sera (S antibodies), SM4s, (+ +) SM4s in sphingomyelin/cholesterol membrane; P antibodies, (+ +) SM4s in dipalmitoyl phosphatidylcholine/cholesterol membrane[534]
Sera (IgG_1) of patients with HIV, TLC-OL, (+) SM4s, SM4g, SM3; (−), lyso-SM4s, SMUnLc$_4$Cer; immunohistochemistry, (+) oligodendroglial cells[87]
Sera of patients with cytomegalovirus (CMV) infection (IgG and IgM), TLC-OL, (+ + + high affinity) SMUnLc$_6$Cer, (+ + moderate affinity) SMUnLc$_4$Cer; (+ low affinity) 0.4 nmol SM4s, 0.5 nmol SM4g, 0.4 nmol SM3; (−) 20 nmol HSO_3-Chol[435]
Sera from patients with autoimmune chronic active hepatitis (IgG), ELISA, (+) SM4s, SM4s-6[593]
Sera of patients infected with *Trypanosoma cruzi* (chronic chagasic disease) (IgA and IgM), ELISA, (+) HSO_3-Chol, 16% in healthy controls and 78% in carriers; (−) dehydroepiandrosterone sulfate[20]
Sera of patients with selective IgA deficiency, ELISA, (+) SM4s (32%), SMnLc$_4$Cer (11%)[28]
Sera of patients with leprosy (IgM), (+) SM4s, (−) HSO_3-Chol[627]
Sera of patients with Guillain-Barré syndrome, (+), SM4s;[122,123,222,435] SBA, TLC-OL, (+) SM4s (43%), diphosphatidylglycerol (48%), SMUnLc$_4$Cer and GM3 (11%)[587]
Sera of patients with inflammatory demyelinating polyradiculoneuropathy, (+) SM4s, nLc$_4$Cer ^{3}IV-NeuAc.[123] Sera from patients of inflammatory polyneuropathy after heart transplantation (IgM), (+) SM4s, GM1[8]
Sera of patients with predominantly sensory neuropathy, TLC-OL, ELISA, immunofluorescence microscopy, (+) SM4s, rat dorsal root ganglia neurons, human neuroblastoma[334]
Sera from patients with autoimmune rheumatic diseases, (+) SM4s (absorbed by DNA, dextran sulfate, heparan sulfate and other anionic molecules)[14]

—*continued*

Table 1—*continued*

Monoclonal
224-58 (IgM-κ), antigen: human brain myelin, ELISA, TLC-OL, (+) SM4s, SM4g, human Schwann cell membrane; (–) GalCer, gangliosides.[150] (+) SM4g, SM4s, the apical ridge of boar spermatozoa; (–) SQ-A_2Gro[135]
2H12, JEG-3 human choriocarcinoma cell line, TLC-OL, ELISA, (+) SM4s, SM4g[600]
412, ELISA, (++) SMUnLc$_4$Cer, (+) nLc$_4$Cer, (–) HSO_3-3GlcUCer.[503] (+) HNK-1 neoglycoprotein[163]
4A9E10 (IgG3), PLC/PRF/5 human hepatoma cells, TLC-OL, ELISA, cytofluorometry, (+) SB1a; (–) SM4s, SM3, SM2a, SB2, gangliosides. 2H6G5 (IgM), PLC/PRF/5, (+) SB1a; (±) SM3. 49-D6 (IgM), HepG2 hepatocellular carcinoma cells, TLC, ELISA, cytofluorometry, (+) SM3, SB1a. 7-E10 (IgM), HepG2, TLC, ELISA, (+) SM3, SB1a. 34-A4 (IgM), PLC/PRF/5, TLC, ELISA, (+) SM3, SB1a;[204] (+) SM4s[200]
AGB43 (mouse IgG3κ), SM4s, TLC-OL, ELISA, (+) SM4s, SM4g, SM3; (–) SM2a, SB2, SB1a, HSO_3-Chol, gangliosides[309,387,538]
AIC3IA2 (mouse IgG3), SM4s, (+) SM4s; (–) GalCer[206]
AMR 20 (mouse IgM), GalCer, TLC-OL, ELISA, (+) GalCer, SM4s, SM4g, SM3, proteoglycans; (–) SM2a, SB2, SB1a, other neutral glycolipids, gangliosides[309]
BMMK-33G (produced by lymphocytes of a breast cancer patient), TLC, ELISA, immunohistochemistry, (+) SM4s, human mammary glands, and other glandular epithelial cells[22]
CA 10 (IgM), SM4g, (+) SM4g, mouse spermatozoa, (–) SM4s, lyso-SM4s[97]
HF2-1/17 (human IgM from a lupus patient), TLC-OL, (+) SM4s, DNA[394]
HNK-1 (mouse IgM), human T cell line HSB-2,[249] (++) SMUnLc$_4$Cer, (+) SMUnLc$_6$Cer.[249,381] IgM paraproteins from demyelinating polyneuropathy patients, ELISA, TLC-OL, (+) SMUnLc$_4$Cer, SMUnLc$_6$Cer, myelin-associated glycoprotein (MAG)[221]
L2 (334) (rat IgM), TLC-OL, (+) SMUnLc$_4$Cer.[221] L9 (mouse IgM), acidic glycolipid mixture from the electric organ of *Torpedo marmorata* (Elasmobranchii), ELISA, TLC-OL, immunohistochemistry, (+) SMUnLc$_4$Cer, SMUnLc$_6$Cer, neuronal cell bodies[44]
M14-376 (human IgM), human lung cancer, TLC-OL, ELISA, immunohistochemistry, (+) SM4s, SM4g, kidney, testis, brain; (–) SM4s-Glc, lyso-SM4s, lyso-SM4g, SM3, SM2a, SM1a, SB2, SB1a, erythrocytes, granulocytes, lymphocytes[378]
NGR50 (mouse IgG2a), SBA, (+) SMUnLc$_4$- and SMUnLc$_6$Cer (20 ng), human MAG, peripheral nerve; (–) rat MAG[642]
O4 (mouse IgM), bovine corpus callosum, (+) SM4s, oligodendrocytes;[521,543] (–) GalCer, Gal-A_2Gro.[521] ELISA, (+) SM4s, SM4g, oligodendrocytes; (±) cholesterol, HSO_3-Chol; (–) SMUnLc$_4$Cer.[27] (+) SM4g, SM4s, lyso-SM4g; (–) SQ-A_2Gro.[135] Immunohistochemistry, (+) Schwann cell lines;[594] (+) low grade and anaplastic astrocytomas;[346] (+) human oligodendrocytes, (–) human astrocytes from a 12-week fetus.[500]
P3 (mouse IgM), GM3(NeuGc), SBA, TLC-OL, (+) NeuGc-containing gangliosides, SM4s, SM1b, human breast tumors[614]
OL-1 (rat mAb), (+) oligodendrocyte culture[127]
R (originally called 'mGalC', mouse IgG3), synaptic plasma membrane preparation, ELISA, (+) SM4s, SM4g, GalCer, Galβ-A_2Gro, GalSph, rat oligodendrocytes and Schwann cells; (–) SMUnLc$_4$Cer.[27] TLC-OL, (+) HT-29, Caco-2 cells.[103] (+) SM4s, SM4g, GalCer, Gal-EAGro; (–) SQ-A_2Gro[135]
SNH1 (mouse Ig), acidic glycolipid extract of melanoma (an upper phase of Folch partition), ELISA, TLC-OL, (+) SM4s, SM4g, SM3, SM2a, SB2, SB1a; (–) HSO_3-Chol, gangliosides, acidic phospholipids; I (+), SM4s; (+) tissues of neuroectodermal and hematopoietic origin, I (+), SM4s.[509]
Sulf I (mouse IgG1), SM4s, TLC-OL, ELISA (+) SM4s, lyso-SM4s, SM4g, lyso-SM4g, SM3; (–) SM4s-Glc,[217] SM2a, SB2, SB1a, glycosaminoglycans, sulfated glycoproteins;[84,125] (+) neutrophils.[17] (+) A and B cells of Langerhans islets, neutrophils and glandular epithelial tissues[56]
VESP 6.2, *Trypanosoma vespertilionis*, TLC-OL, ELISA, (+) SM4s[451]

Symbols and abbreviations: (+), positive interaction; (–), interaction not detected; I (+), inhibition of interaction; I (–), no inhibition of interaction; SBA, solid-phase binding assay; TLC-OL, TLC overlay; ELISA, enzyme-linked immunosorbent assay

Table 2. Molecules and cells interacting with sulfatides

Prokaryotes
Herpes simplex virus (HSV)-1, glycoprotein C, (+) SM4s, heparan sulfate[208]
Human immunodeficiency virus (HIV), envelope protein gp120, TLC-OL and SBA, (+) SM4s, GalCer; (–) GlcCer, gangliosides.[663] TLC-OL using ^{125}I-gp120, (+) GalCer, GalSph, GM4, SM4s (5–10 nmol).[36] ELISA, (+) SM4s, IV^3-NeuAc-nLc$_4$Cer, GalCer, myelin-associated glycoprotein (MAG); (–) chondroitin sulfate, heparan sulfate.[656] TLC-OL (gp120), (+) GalCer, human colonic epithelial cell lines H-29 and Caco-2;[103] (+) SQ-A_2Gro (0.2–1.0 μg/ml);[148] (+) SM4g.[618] Gp120, ELISA and by an immunosorbent assay on nitrocellulose paper, (+) SM4s, (–) GalCer, GM1; an immunosorbent assay on TLC plates, (+) GalCer, SM4s, GM1; (+) SM4s incorporated into the plasma membrane of lymphoid cells[662]
Influenza virus, TLC-OL, (++) SM4s, GalCer, GD1a, nLc$_4$Cer IV^3-NeuAc; (+) LacCer; (–) lyso-SM4s, GM3-NeuGc[549]
M. pneumoniae cell, SBA, (+) SM4s, SM4g, SM3, laminins, glycoproteins; (–) gangliosides, neutral glycolipids; I (+) dextran sulfate, 3′-sialyllactose, human colon adenocarcinoma cell line (WiDr)[314,470]

—*continued*

Table 2—*continued*

M. pulmonis (rodents), TLC-OL, (+) SM4g, SM4s; (–) NeuAc-3LacNAc; I (+) dextran sulfate; I (–) SM4s, NeuAc-3LacNAc.[355] *M. hominis*, *Ureaplasma urealyticum* (humans), *Ureaplasma diversum* (cattle), TLC-OL, (+) SM4g, SM4s.[354] *M. hominis* (humans), TLC-OL, (+) SM4g (2 nmol), SM4s; SBA > 0.1 nmol/well, colon carcinoma cell line WiDr; I (+) dextran sulfate; I (–) 10^{-2} M NaCl, HSO_3-Chol[444]

M. hyopneumoniae, SBA, TLC-OL, (+) SM4s, GM3, Gb_4Cer, cilia and ciliated cells of swine respiratory epithelia; (–) HSO_3-Chol; I (+), heparin[652]

Bordetella pertussis (virulent and avirulent strain), TLC-OL, (+) GalNAcβ4Gal-R (e.g. asialo GM2); (–) GM1, Gb_4Cer; *B. pertussis* (virulent strain), TLC-OL, (+) SM4s; I (+) dextran sulfate, fucoidan; (+) human colon adenocarcinoma cell (WiDr), I (+) heparin.[48] TLC-OL, (+) SM4s, (–) gangliosides, neutral glycolipids, a heparan sulfate proteoglycan[172]

Escherichia coli, S-fimbriated, TLC-OL, (+) SM4s, SM4g, GalCer, LacCer; (–) gangliosides and other neutral glycolipids.[454] *E. coli*, 987P fimbriae, TLC-OL, (+) SM4s (d18:1/hydroxy fatty acids, < 5 nmol), pig intestine.[283]

Helicobacter pylori, TLC-OL, (+) SM4s, SM3, GM3; flow cytometry, a gastric cancer cell line Kato III; (–) neutral glycolipids[259]

Helicobacter pylori, neutrophil-activating protein, TLC-OL, SBA, (+ + +) SM4s, SM1b, NeuAc(or NeuGc)nLc_4 or $_6$Cer; (–) neutral glycolipids, ganglio-series gangliosides[589]

Tetanus toxin, TLC-OL, (+) SM4s, SCLC (small cell lung cancer) cell lines; (–) lung tissue[144]

Eukaryotes

Arthrobotrys oligospora lectin, TLC-OL, SBA, (+) SM4s, SM3, SM1b, HSO_3-Gal-ManGlcE_2Gro, acidic phospholipids[476]

Malaria circumsporozoite protein, the carboxy terminal region II, binding to sulfated glycoconjugate-Sepharose, (+) heparin, fucoidan, dextran sulfate; SBA, (+) SM4s, (±) HSO_3-Chol; binding of ^{125}I-protein, (+) hepatocytes;[446] (+) SM4s, HSO_3-Chol.[62] (+) SM4s[390]

Avian and human malaria plasmodium sporozoite, genes of thrombospondin-related anonymous protein (TRAP), contained sequences corresponding to thrombospondin-like sulfatide binding domain[585]

Antistasin (a salivary protein from the Mexican leech *Haementeria officinalis*), (+) SM4s, sulfated glycosaminoglycans; (–) HSO_3-Chol, gangliosides; I (+) dextran sulfate, heparin[208]

Melittin (a toxic bee peptide), (+) SM4s[114]

Laminins, TLC-OL, SBA, (+) SM4s, SM4g, SM4s-6, SM3, SM2a, SB2, SB1a; I (+) fucoidan, dextran sulfate; I (–) chondroitin sulfate, hyaluronate, colominic acid, yeast phosphomannan; (–) neutral glycolipids, gangliosides.[471] The globular carboxyl domain of laminin α chain, SBA, (+) SM4s, heparin; after reduction and alkylation, (–) SM4s.[583] SBA, TLC-OL, (+) SM4s, SM4g, SMUnLc_4Cer, SMUnLc_6Cer; I (+) heparin.[381] TLC-OL, (+) SM4s, SM3, SB2, heparin, heparan sulfate; erythrocytes (via SM4s at RBC surface membrane).[472] TLC-OL, (+) SM4s, SM4g, melanoma cell lines; (–) HSO_3-3Chol; SBA, I (+) for heparin, I (–) for SM4s, synthetic peptides from a thrombospondin type I repeats (e.g. KRFKQDGGWSHWSPWSS) containing WSPW sequence.[157] (+), heparin, through the carboxyl terminal X-B-B-X-B-X, and B-X-B-X-B-X sequences.[61] Laminin-1, SBA, electron microscopy, (+) a HNK-1 neoglycoprotein (through E8) (binding not abolished by reduction and alkylation or by urea treatment); heparin; I (+) heparin; I (–) SM4s.[163] E3 fragment of domain G4-G5, (+) SM4s, heparin; (–) a HNK-1 neoglycoprotein.[163] Laminin, (+) SMKT-R3 renal carcinoma cells, I (+) anti-laminin antibody, monoclonal antibody Sulf-I.[294] TLC-OL, (+) SMUnLc_4Cer, mouse neurons and astrocytes.[503] Laminin-1, laminin-2/laminin-4 and E8 fragment, mouse cerebellar neurons, cell adhesion (+); urea denatured E8, adhesion (–).[163] Laminin binding with α-dystroglycan or a schwannoma cell line RT4, I (+) SM4s, heparin, EDTA; I (–) chondroitin sulfate.[372]

Thrombospondins, TLC-OL, SBA, (+) SM4s, SM4s-6, SM3, SB2, SB1a, SM2a, heparin, fucoidan, (–) HSO_3-Chol; I (+) fucoidan, dextran sulfate, heparin;[471] (+) SMUnLc_4Cer, human melanoma cells.[469] TLC-OL, (+) SMGb_4Cer;[400] (+) SMGb_5Cer.[399] (+) SM4s, heparin, melanoma cells; I (+) for heparin, I (–) for SM4s, synthetic peptides from a thrombospondin type I repeats (e.g. KRFKQDGGWSHWSPWSS).[157] Thrombospondin, and laminin, TLC-OL, SBA, (+) SM4s; dynamic flow adhesion assay, (+) erythrocytes; I (+) high molecular weight dextran sulfate, chondroitin sulfate A[200]

Von Willebrand factor (high molecular coagulation factor VIII), (+) SM4s, SM4s-6, SM3, SB2, SB1a; I (+) dextran sulfate, I (–) other glycosaminoglycans.[471] (+) Human platelet;[83] rat and human platelets, 164–512 pmol/10^9 platelets.[40] (+) SM4s, binds A1 domain.[73] Recepter binding, I (+) sulfobacins A and B, and SQ-A_2Gro from a gliding bacterium[260]

Properdin (human serum), SBA, (+) SM4s (> 62.5 nmol) and other sulfated glycosaminoglycans; (–), gangliosides, HSO_3-Chol, phospholipids; I (+) dextran sulfate, fucoidan.[198,209] TLC-OL, ELISA, SM4g, SM4s (> 1 nmol), SM3, SB2, SM2a, SMUnLc_4Cer; (–) GM1, GD3; I (+) dextran sulfate.[246]

Vitronectin (mammalian blood plasma), the secondary structure prediction method, heparin, through X-B-B-X-B-X, and X-B-B-B-X-X-B-X sequences.[61] SBA, (+) SM4s, HSO_3-Chol; (–) gangliosides; I for SM4s binding, (+) HSO_3^-, fucoidan, dextran sulfate; (–) heparin, heparan sulfate; I for HSO_3-Chol binding, (–), HSO_3^-, 1 M NaCl[646]

Fibronectin (an extracellular matrix protein), SBA, (+) heparin; (–) SM4s.[583] SBA and cell lytic assay, (+) SMKT-R3 cells, I (–) anti-laminin antibody[294]

Amyloid P + Ca^{2+} (human serum), TLC-OL, (+) all sulfatides tested (0.1–5 nmol), GAG, mannose 6-phosphate; I (+) EDTA, glucose 6-phosphate, SM4s (5 μM); I (–), lyso-SM4s[357]

Apolipoprotein E, (+) SM4s.[157] The secondary structure prediction method, (+) heparin, through X-B-B-X-B-X, and X-B-B-B-X-X-B-X sequences.[61] (+) SM4g; I (+) synthetic peptides from a thrombospondin containing WSPW (e.g. KRFKQDGGWSHWSPWSS)[157]

—*continued*

Table 2—*continued*

Fibrinogen; (+) SM4s in 1:400 molar ratio[179]
High molecular weight kininogen (participates in the intrinsic phase of blood coagulation as the complex with human factor XI), (+) SM4s, dextran sulfate, negatively charged surfaces (through domain 5)[584]
Human factor-H, TLC-OL, SBA, (+) SB2 (1 nmol), SM4s, SM4g, SM3[246]
Multicatalytic proteinase, human erythrocytes, TLC-OL using ^{125}I-labeled enzyme, (+) SM4s, SM3; (–) neutral glycolipids, gangliosides[438]
NKR-P1 protein (natural killer cells), (+) heparin hexasulfotetrasaccharide > heparin tris-sulfodisaccharide > heparin *bis*-sulfodisaccharide > heparin monosulfodisaccharide > chondroitin 6-sulfate monosulfodisaccharide > chondroitin 4-sulfate monosulfodisaccharide > 3′-sulfated Le^a pentasaccharide > SB2 > SM2 > SB1a, SMUnLc$_4$Cer > SM1a > SM3 > SM4s in the order of larger amount of the protein bound (oligosaccharides as neoglycolipids).[35] (+) HSO$_3$-3Galβ-3(Fucα-4)-GlcNAcβ-3Gal-4Glc-neoglycolipid[298]
Human platelet, (+) SM4s; (–) HSO$_3$-Chol, gangliosides; I (+) von Willebrand factor, dextran sulfate, I (–), heparin[83]
E-selectin + Ca^{2+}, (–) SM4s.[169] E-selectin + Ca^{2+}, TLC-OL, (+) HSO$_3$-3Galβ-4(Fucα-4)GlcNAcCer; HSO$_3$-3-Galβ-4(Fucα-3)GlcNAc-1Cer.[112] TLC-OL with E-selectin transfected CHO cells, (+) HSO$_3$-3Galβ-3(Fucα-4)-GlcNAcβ-3Gal-4Glc-neoglycolipid.[298] E-selectin-IgG + Ca^{2+}, SBA, (+) 2,3-sialyl Le^x, SM4s; I (+) EDTA; (–) SMUnLc$_4$Cer.[416] Chimeras possessing the carbohydrate recognition domain of E-selectin, SBA, (–) SM4s.[301] E-selectin with substitution of EGF domain residues 124 and 128, SBA, (+) SM4s, HL60 cells[467]
L-selectin-IgG + Ca^{2+}, TLC-OL, SBA, (+) SM4s, SM3, SB2, SB1a, sulfated fucans, SM4s-6, (±) HSO$_3$-Chol; (–) SM1a; I (+) SM4s, heparin.[223] TLC-OL, SBA, (+) SM4s, SMUnLc$_4$Cer, heparin; I (–) EGTA; (+) sulfated Le^a/Le^x tetrasaccharides; I (+) EGTA.[153] L-selectin-IgG, TLC-OL, ELISA (30–100 pmol), (+) SM4s (50% of the interaction with sulfatides can be Ca^{2+}-independent), SM4g, SM3, SM2a (weak), SB2, SB1a, synthetic sulfatide analogues; Sia-Le^a; (–) gangliosides, neutral glycolipids.[550] (+) HSO$_3$-3Galβ-3(Fucα-4)-GlcNAcβ-3Gal-4Glc-neoglycolipid.[298] L-selectin-IgG + Ca^{2+}, ELISA, (+) 2,3-sialyl Le^x; (–) octadecyl sulfate, sphingosine sulfate; L-selectin-IgG + Ca^{2+}, (+) SM4s, SMUnLc$_4$Cer, desulfated SMUnLc$_4$Cer; I (–) EDTA.[417] L-selectin-IgG + Ca^{2+}, SBA, (+) HEV-derived cell line, Ax.[581] The carbohydrate recognition domain of L-selectin, (+) SM4s[301]
P-selectin-Ig + Ca^{2+}, TLC-OL or ELISA, (+) SM4s, (–) Lyso-SM4s; I (+) SM4s, sulfated glycosaminoglycans; flow cytometry, granulocytes' lipids; I (+) SM4s.[17] P-selectin-Ig + Ca^{2+}, flow cytometry or SBA, (+) SM4s; I (+) heparin.[23] P-selectin-IgG + Ca^{2+}, (+) sialyl Le^x, SM4s, SMUnLc$_4$Cer, desulfated SMUnLc$_4$Cer; I (–) EDTA; (–) octadecyl sulfate, sphingosine sulfate[417]
Saposin B (sap-B), human, release of ^{35}S, (+) SM4s, lyso-SM4s, SM4g, lyso-SM4g.[115] Tyrosine fluorescence measurement, (+) SM4s, Lyso-SM4s, lyso-SM4g, GM1, Gb$_3$Cer, sphingomyelin.[330] TLC-OL, SBA, (+) SM4s, GM1.[202] (+) GalCer, GlcCer, SM4s; I (+) Mg^{2+}, Ca^{2+}, Zn^{2+}.[531] TLC-OL, (+) SM4s, neutral glycolipids, gangliosides[164]
Saposin C (sap-C), human, TLC-OL using each 15 nmol glycolipid, (+) SM4s, SM3, GM4, GM3, GM1, GM2, GalCer, LacCer, Gg$_3$Cer.[164]
Tamm-Horsfall protein, (+) SM4s[648]
SLIP 1 (sulfolipid-immobilizing protein 1 isolated from male germ cells), (+) SM4g, SM4s[350]
Opioid peptides, (+) SM4s.[356] Morphine, (+) phosphatidylserine.[4] Levorphanol, (+) SM4s with hydroxy fatty acid (Kd 9.1×10^{-8}), SM4s with nonhydroxy fatty acid (Kd 1×10^{-6})[356]
Myelin basic protein, (+) SM4s[114,493]
Amphoterin (a 30 kDa protein from rat brain), TLC-OL, SBA, (+) SM4s, SM4g, SMUnLc$_4$Cer, SMUnLc$_6$Cer, heparin, fucoidan; (–) bile acid sulfate, gangliosides, desulfated SMUnLc$_4$Cer[382,405]
Hepatic growth factor (HGF), ^{125}I-HGF-OL, (+) SM4s, SM3, SB2, heparin, renal cell carcinoma (Grawitz) cell line SMKT-R3.[291] (–) SM2a, neutral glycolipids, gangliosides; I (+) dextran sulfate, heparin, fucoidan; I (–) keratan sulfate, hyaluronic acid[295]
Cytotactin (tenascin, a glial glycoprotein), SBA using radiolabeled cytotactin, SM4s (> 4 pmol); I (+) EDTA. (+) SM4s, HNK-1[79]
Rat Schwann cells, TLC-OL, (+) SMUnLc$_4$Cer, SMUnLc$_6$Cer; (–) gangliosides.[416] Rat peripheral nerve myelin, TLC-OL, (+) SMUnLc$_4$ covalently attached to bovine serum albumin (BSA) via reductive amination; I (+) SMUnLc$_4$Cer[507]
Human melanoma cell line G361 (laminin-independent), SBA, (+) SM4s, SM4g > 100 pmol/cm^2; I (+) fucoidan; (laminin-dependent) (+) SM4s, SM4g > 2.5 pmol/cm^2; (±)HSO$_3$-Chol; I (+) fucoidan, dextran sulfate, anti-laminin antibody, anti-laminin-receptor polyclonal antibody, thrombospondins; (–) neutral glycolipids, gangliosides[472]
Human C32 melanoma cells, SBA, (+) SM4s, SM4g; I (+) thrombospondins, fucoidan, dextran sulfate; I (–) laminins, HSO$_3$-Chol[472]
Human melanoma cells, (+) thrombospondins[469]

Symbols and abbreviations: (+), positive interaction; (–), interaction not detected; I (+), inhibition of binding; I (–), no inhibition of binding; TLC-OL, TLC overlay; SBA, solid-phase binding assay; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay

labeled sulfolipids, and the data can be transferred through a network to personal computers, analyzed for images and incorporation quantitated and calculated.

(iv) TLC overlay. Immunostaining using monoclonal antibodies[121,125] against sulfatides (Table 1) and overlay with adhesive proteins,[468,581,613] lectins, bacteria and

viri[263,391] (Table 2) are able to specifically locate individual sulfatide on TLC plates. Conversely, ligand specificities of the carbohydrate-binding proteins can be determined using synthetic neoglycolipids as immobilized probes on TLC plates.[112] The TLC overlay's potential disadvantage is that many monoclonal antibodies recognize antigens in unnaturally high densities but often are not capable of reacting with the same antigen expressed on the cell surface in low density.[80,161,613] With Sulf I monoclonal antibody the lowest detectable amount of SM4s and SM3 on TLC was 5 pmol, whereas the lowest limit for SM4g was only 1 pmol.[125] The half-maximal binding was 1–3 pmol per well with SM4s on microtiter well while lyso-SM4g and lyso-SM4s showed very low sensitivities (>1.5 nmol). After the washing and staining procedures used for the assay, only 36% of the standard [^{14}C]-labeled SM4s remained on microtiter wells, and with lyso-SM4s, 50%,[477] or even only 7%[125] of the ^{3}H radioactivities remained indicating that the low sensitivities were due to the loss from the wells.

SMGb$_5$Cer and SMGb$_4$Cer were stained with the monoclonal anti-SSEA-3 antibody that interacted with the internal structure, R-3GalNAcβ-3Galα-4Galβ-R'. The structure 'R', HSO$_3$- of SMGb$_4$Cer and HSO$_3$-3Galβ- of SMGb$_5$Cer, did not interfere with the recognition by this antibody,[399,400] while the monoclonal anti-SSEA-4 antibody, whose epitope was reported to be NeuAc2-3Galβ-3GalNAcβ-R, did not interact with SMGb$_4$Cer and SMGb$_5$Cer.[400] This may indicate that a NeuAc residue on the terminal Gal of Gb$_5$Cer was essential for antibody binding and that a sulfate ester was not able to replace the sialic acid. Bands of SM4s (>8–11 pmol) were detected with ^{125}I-labeled thrombospondins[468] or hepatic growth factor (HGF)[295] on TLC. The staining of SMGb$_4$Cer by thrombospondins was only 0.001 in comparison to the staining of SM4s.[400] The bacterial-binding assay on TLC has the advantage that TLC plates present glycolipid receptors in a conformation similar to that of the eukaryotic cell membrane[263] and discriminate the pathogenic prokaryotes using lipids containing SM4s from the tissue of 5 mg dry weight (Table 2).[259]

2. *Ion-exchange Chromatography*

Anion exchange column supports used at present include DEAE–cellulose,[415,479,480] TEAE–cellulose,[119,479] DEAE–Sephadex,[217,385,559,567] DEAE–Sephacel,[233] DEAE–Sepharose 6B,[200] DEAE–Toyopearl,[218,565,568] and QAE-Sephadex.[624] Column chromatographies of anion exchangers have been routinely used for the initial phase of the purification of acidic lipids, including sulfatides (Figs 2 and 3), plant SQ-A$_2$Gro, sulfolipids of mycobacteria,[149] and halophilic archaea.[273]

Anionic lipids elute from DEAE–Sephadex columns with chloroform/methanol/ammonium acetate (5:10:1) system in the order of: (1) diphosphatidylglycerol;[233] (2) monosialosyl gangliosides and free fatty acids; (3) monosulfoglycolipids (SM2a, SMiGb$_4$Cer/SMiGb$_5$Cer, SM2b, SM4s (d18:1/24:0), SM3 (d18:1/24:1), SM4s (d18:1/24h:0), SM4s-Glc (t18:0/24:0) in this order);[217,233,564,565,568] (4) disialosyl gangliosides (GD3 and GD1a); (5) HSO$_3$-Chol and SM4s-Glc (t18:0/2-hydroxy fatty acids); (6) monosialosyl monosulfoglycolipid (SMGM1a);[567] (7) *bis*-sulfated glycolipids (SB2 and SB1a);[561,567] and (8) trisialosyl, and tetrasialosylgangliosides, and SMUnLc$_4$Cer.[68,221,416] Sulfatides containing an *N*-acetylhexosamine eluted earlier than those composed of only hexoses.[559] DEAE–Sephadex but not DEAE–Sephacel resolves SB2 and SB1a because the elution profiles are to some extent modified by the support (Sephadex, Sepharose, and Toyopearl) and the solvent (chloroform/methanol/water or methanol). SM4s-Glc (t18:0/hydroxy fatty acid) eluted later than HSO$_3$-Chol probably because glucose is adsorbed to Sephadex stronger than galactose. SM3[204] and SMUnLc$_4$Cer[221] were effectively purified by elution with ammonium acetate in methanol.

Neutral lipids (e.g. cholesterol, and methyl esters of fatty acids released from acylglycerolipids) and zwitterionic phospholipids (e.g. sphingomyelin, and lyso-phosphatidyl-

choline) are collected in the pass-through fraction. Acidic phospholipids including diphosphatidylglycerol, and phosphatidylserine, phosphatidylinositol and their lyso-derivatives, eluted from the DEAE column along with monosulfoglycolipids (Fig. 2). The recoveries of SM4s from anion exchange columns ranged from 93[228] to 95%.[279] The assay mixture of GalCer sulfotransferase was first washed with Folch's partition and then applied to a small DEAE–Sephadex column to remove residual [^{35}S]sulfate, [^{35}S]PAPS and [^{3}H]GalCer.[137,279]

Acidic oligosaccharides, derived from SM4s[483] or SMUnLc$_4$Cer[163,416] by treatment with endoglycoceramidase, or ceramide glycanase respectively, were separated and quantitated using a Dionex[483] or a Dowex column.[163] Three to five μg of SM4s was sufficient for the detection of the peak of the released HSO$_3$-Gal by pulsed amperometry.[483]

3. *Adsorption Column Chromatography*

Silicic acid is a potent adsorbent for acidic lipids of a wide polarity range.[91,479] The water in chloroform/methanol system attaches the effect of partition to the primarily adsorptive nature of silicic acids. SM4s and SM4g eluted usually with chloroform/methanol/water mixtures[84,318,412,558] closely after HSO$_3$-Chol.[233,559] A rat brain lipid mixture was able to be separated further into SM4g ($E_{16:0}A_{16:0}$), SM4s with nonhydroxy fatty acids and SM4s with hydroxy fatty acids (d18:1/24h:0) on a silica bead (Iatrobeads) (ϕ 60 μm) column[228] (recovery, 88%). SM4s[445] and more complex sulfatides, SM2a, SM2b, SB2, SB1a, SMiGb$_4$Cer, SMiGb$_5$Cer,[565,568] and SMGM1a[567] were purified by HPLC using silica bead columns of 5–10 μm diameter. These sulfatides with the ceramide composed of d18:1 and nonhydroxy fatty acids usually elute significantly faster than those with an identical polar group but containing t18:0 and 2-hydroxy fatty acids.[565]

The salt form may change after passing through a silicic acid column because silicic acids contain cations including Na^+, K^+, Ca^{2+}, and Mg^{2+}.[159] Preequilibration with dilute salt solution may lead to a better separation of peaks because silicic acids have mild ion-exchange capacity[236] and the addition of NaHCO$_3$, or KCl in the elution solvent may reduce the adsorptive strength resulting in better recoveries of sulfatides. Separation of highly polar sulfatides[401,407] including SMiGb$_5$Cer,[564] SMGM1a,[567] and HSO$_3$-8NeuGc2-6GlcCer[315] was also improved by using solvents containing up to 4 M of NH$_4$OH. For purification of radioactivity-labeled sulfatides in the total lipid extract from a few milligrams of tissues, nonradioactive SM4s[32,521] or SM2a[230] was added as the carrier to guarantee high recoveries.

Chloroform/acetone systems have been used for the purification of less polar glycolipids from prokaryotes, plants, and animal brain because SM4g,[95,116,232,306,381,428,511] SM4s,[608] and HSO$_3$-2Man-4Glc-E$_2$Gro[597,598] show stronger affinity to acetone[232] than phospholipids. After washing out the acyl glycerides and cholesterol with chloroform, the glycolipids may be eluted from the column with solvent mixtures containing the increasing concentration of acetone in chloroform or dichloromethane, culminating with pure acetone[32,288] or acetone/methanol, 9:1.[110,610] Care must be taken in the formation of isopropylidene derivatives of galactolipids by acetone,[317] especially when the elution medium is acidic.[349] HPLC using the combination of porous silica columns and solvents such as 2-propanol in hexane allows rapid separation of brain glycolipids with high resolution.[88]

Florisil (aluminum silicate) has been a convenient column support to purify SM4s,[180,278,480,620,621] SM3,[491] and HSO$_3$-Chol[39,647] with reasonable recoveries (e.g. SM4s, 85.5–87.0%[282]), while for more polar sulfatides, the adsorptive capacity of Florisil is too strong even after deactivation by the addition of 1–7% of water.[189,232] Use of paper or cellulose column chromatography for purification of SM4s was not successful.[162]

Sulfatides were purified from the acidic glycolipids of platelets[394] and the total lipid extracts from human spermatozoa[11] by the column of aminopropyl-bonded silica gel with recoveries between 89 and 98%. SMUnLc$_4$Cer and SMUnLc$_6$Cer were separated

from each other by HPLC with a Licrosorb–NH_2 column.[68] Florisil or silicic acids have been used as the adsorbent to separate peracetylated SM4s,[486] SM4g,[606] and SM1b.[47] Acetylation of hydroxyls drastically reduces the polarity of sulfatides and improves the recovery from the column. For acetylation, sulfatides should be incubated with acetic anhydride/pyridine at ambient temperature[563] to prevent desulfation involving electrophilic attack by the acetylium ion ($CH_3C^+{=}O$) leading to scission of the S—O bond.[603]

A mixture of cholesterol, HSO_3-Chol, GalCer and SM4s was benzoylated to convert polar lipids except for HSO_3-Chol into nonpolar benzoate derivatives.[243] HPLC determination of benzoylated and desulfated SM4s[88,415,515,521] as well as perbenzoylated SM4s, SM4g, and lyso-SM4g[529] have been reported. The recoveries of SM4s from rat spinal cord[88] and human urine[415] were 50–60%, and 30% respectively. The overall recovery of benzoylation/debenzoylation method of SM4s was 72%.[544]

The cerebellar amphoterin[382,405] and anti-sulfatide antibodies[435] were purified on affinity columns of SM4s–octyl–Sepharose. Purified SM4g was coupled to aminopropylglass with a photoactivated, heterobifunctional crosslinking agent, *N*-hydroxy-succinimidyl-azidobenzoate and used for the purification of antibodies.[97]

III. ANALYTICAL METHODS

The selection of appropriate analytical methods, and reasonable recoveries in purification steps are essential to obtain reliable values for sulfatides as exemplified in the reports with widely different concentrations on the same tissue (Table 2). Lipid composition data should preferably be described in moles per unit fresh weight because direct comparison of lipid classes and sulfoamphiphiles become possible by using this mode of expression.[480]

A. Colorimetric Analysis

1. Chemical Analysis

Conventionally, inorganic sulfate released from sulfatides has been quantitated chemically[481] but recently the assay of either the chromogenic ion pair formed between sulfatides and Azure A,[282] or the chromogen or radioactivities obtained by the solid phase binding of specific antibodies or adhesive proteins are preferred.

(a) Sulfate

The assay of inorganic sulfate liberated from sulfoconjugates, as the precipitates of barium salts or by chelating barium ions with rhodizonate,[482] is reliable but laborious and not satisfactory in sensitivity.[481] Recently, an HPLC analysis of sulfate ion using 5–10 nmol of sulfatides was reported.[218]

(i) The *rhodizonate method.* The inorganic sulfate, released from sulfatide by HCl hydrolysis,[232,588] Schöniger's oxygen combustion method[647] or enzyme-catalyzed hydrolysis,[482] chelates with barium rhodizonate resulting in the destruction of the chromogen, which is assayed colorimetrically with the sensitivity of 1–10 nmol.[588] Only oxidative mineralization in boiling HNO_3 released the sulfate from SQ-A_2Gro[272] because the negatively charged oxygen shields the carbon atom of the sulfonic acid from attacks by negative groups (e.g. Cl^-).[159,332]

(ii) *Azure A.* The colored ion pair formation of the cationic dye, methylene blue, had been used primarily for the determination of relatively nonpolar sulfatides[159,481] and later, and this assay method was modified for SM4s using Azure A as the dye.[282] The ion pair of the hydrophilic Azure A with sulfatides (absorption at 635 nm) is more lipophilic and partitioned into the organic phase of chloroform/methanol/0.1 M H_2SO_4 mixture. Azure A assay is the method of choice for SM4s, SM4g, and SDS for its sensitivity (above 0.5 nmol) and simplicity, although crude lipid preparations may yield optical densities higher than the sulfatide actually con-

tained[519,615] because gangliosides, and acidic phospholipids interact weakly with this pigment.[282] In addition, the partition step involved in the procedure is susceptible to the lipophilicity of the aglycon. For instance, testicular SM4g yielded 1.22-fold of the molar extinction when human kidney SM4s was used as the standard,[563] while more polar sulfatides such as SM3 and SB1a yielded only 0.82- and 0.19-fold respectively. Acetylation of sulfatides circumvented this difficulty.[563] Peracetylated SMGb$_4$Cer,[400] HSO$_3$-6ManGlc-E$_2$Gro,[369] and SMGM1a,[567] for instance, yielded optical densities of 0.097, 0.093, and 0.11/nmol respectively, comparable to that of SM4s, 0.101/nmol. However, caution is necessary to apply peracetylation/Azure A method to an unknown sulfatide because, for instance, (HSO$_3$)$_2$-2,6ManGlc-E$_2$Gro yielded only 1.5-fold absorption in comparison to the reference SM4s. Interestingly, TLC densitometry of the native form of this *bis*-sulfoglycolipid resulted in a value of 2.27 mol sulfate/mol *bis*-sulfoglycolipid.[369]

It should be noted that some lots of Azure A and rhodizonate[481,482] yielded anomalously high background optical densities. The setback of the Azure A method, the possible contamination of the colored organic phase by the upper pigment phase, can be avoided by using a glass micropipette with a screw aspirator or by the use of a new mixture of solvents in which the desired layer floats above the excess dye.[459]

(b) Other constituents

Sulfoglycosphingolipids can be determined by fluorescamine method after hydrolysis of the band on silica gel (sensitivity 0.2 nmol, recovery 70%),[606] or by the orcinol/sulfuric acid assay of silica gel powder containing glycolipids (50–200 nmol).[420] A deep blue color of anthrone sulfuric acid reaction of 6-sulfoquinovose has an absorption maximum at 592 nm while glucose, galactose and glucose 6-sulfate peak at 625 nm. With free galactose liberated by acid hydrolysis of SM4g or SM4s on silica gel powder, the fluorescence intensity of NADH in the assay by β-galactose dehydrogenase was linear from 1 to 6 nmol of galactose.[606] Fatty acids and trimethylsilylated methylglycosides obtained by methanolysis in HCl/methanol were quantitated gaschromatographically using heptadecanoic acid[232] and mannitol respectively, as internal standards,[229] although mannitol should be used with caution for recovery especially when Ag_2CO_3 is used for neutralization.[398] Also, cholesterol released from HSO$_3$-Chol,[217,243,428,580] as well as Gal-1-alkylGro or GalGro[228] from SM4g can be determined using appropriate internal standards.

2. *Antibodies and Adhesive Proteins*

Serum antibodies interacting with sulfatides have been identified in a number of antoimmune diseases and may be responsible for the pathology in some cases.[534] On the other hand, sulfatides can be detected and determined by using artificial monoclonal antibodies[121,204] and specific binding proteins[112,468,550] as listed in Tables 1 and 2. The monoclonal antibodies Sulf I[125] and AGB43[309] recognize SM4s, SM4g, their lyso-derivatives and SM3, while they did not interact with SM4s-Glc,[217] and other mono- and *bis*-sulfo glycolipids with sugar chains longer than disaccharides. M14-376 monoclonal antibody (human IgM) showed most stringent specificity interacting only with SM4s and SM4g.[378] Obviously at least a part of the hydrophobic region of the sulfatides interacts with all the above antibodies.

(a) Solid phase binding assay

SM4s,[122,506] SMUnLc$_4$Cer,[381,417] and HSO$_3$-Chol[20] are adsorbed to plastic wells or silica gel layers in methanol,[406] 50% aqueous solutions of ethanol, or chloroform/methanol and, after evaporation of the solvent, fixed with polyisobutylmethacrylate sometimes with the aid of admixed auxiliary lipids.[27,200,468,468] TLC may, however, be less specific than the other assay systems, as binding on TLC plates can be altered by the polyisobu-

tylmethacrylate coating. Sulfatides are detected by using a ^{125}I-labeled ligand,[468] or indirectly by using peroxidase conjugated secondary antibody[125] specific to the primary antibody, or streptococcal protein A.[204] Microwell- and TLC-immunoassay are two orders of magnitude more sensitive than the conventional chemical methods. For instance, the densitometric response of TLC–ELISA of SM4s was linear between 15 and 250 pmol of SM4s.[84] A control TLC plate treated similarly, but stained by orcinol, showed that substantial amount of sulfatides remained on the plate after the immunostaining procedure.[550] However, several reports cause concern regardng loss by background washing procedures.[153] The recovery of SM4s in microwells of ELISA ranged from 20%[333] to 82%.[381] Even when ^{14}C-labeled sulfatides on microtiter well were incubated only once with Tris/BSA for 15 min, 73% of SM4s and only 13% of lyso-SM4s remained adsorbed to the wells.[125] Sulfatides with nonhydroxy-fatty acids were markedly better retained in microwells than sulfatides with hydroxy-fatty acids.

(b) Histological and cytofluorometric analysis

A considerable portion of polar lipids survives the routine dehydration procedure for paraffin embedding of tissues. Prior treatment of tissue sections with cold acetone, on the contrary, improved the staining.[538] However, stronger extraction procedures using chloroform/methanol demolished the staining with the monoclonal antibodies[44,55,210,309,316] or L-selectin,[581] suggesting that the cross-reactivity of the monoclonal antibodies with glycoproteins may be minimal. Arylsulfatase A activity was stained on frozen sections mounted on slides.[615] Human hepatocellular carcinoma cell lines,[204] and SMKT-R3 renal carcinoma cells[293–295] were analyzed by flow cytometry, after labeling with monoclonal antibodies or anti-laminin IgG then with fluorogenic second antibodies, producing distinct images of the cell populations expressing sulfatides on the cell surface.

B. Spectroscopic Analysis

About 30 years ago a few physical properties, including the melting point, 213°C;[278] optical rotation; and infrared absorption,[637] were the only reliable parameters to identify SM4s. Today only ^{1}H-NMR can establish the full structure except for stereochemistry using a few to several hundred nmol of sulfatides[369,565] and when the amount is still meager, FAB, LSIMS[566] or MALDI-TOF[562] can identify the partial structure in pmol or even fmol.

1. Optical Rotatory Dispersion (ORD)

Other optical rotation data reported for SM4s are, $[\alpha]^{18}{}_{D} = -0.14$ (C = 3.56 in pyridine),[360] $[\alpha]^{24}{}_{D} = +2.84$,[278] and $[\alpha]^{24}{}_{D} = -0.2$ (c = 2.0 in pyridine).[548] ORD used today is much more sensitive and applied to the study of the configuration of component saccharides, e.g. SQ-A_2Gro[518] and HSO_3-2Manα-2Glcα-1$E_{20}E_{20}$Gro.[598] The stereochemistry of the glycerol monoether from SM4g[606] and glycerol tetra-ethers from lipids of a thermophilic archaea[89] was determined by optical rotation.

2. Infrared Spectroscopy

The infrared (IR) absorption was once frequently used for the characterization of sulfate esters in sulfatides,[159,232] lyso-sulfatides[159,307,432,569] and HSO_3-Chol.[39,388,607] Recently, FT–IR apparatus replaced the conventional machines providing a better signal to noise ratio with several times smaller amounts of sulfatides (10–20 nmol).[564,565,568]

The relatively broad absorption of the asymmetric O═S—O$^-$ stretching vibration at 1210–1265 cm^{-1} [68,232,273,275,557,603,637] is intense but overlaps with that of phosphate esters[116,370] (being not obvious, for instance, in the spectrum of HSO_3-PtdGro[167]). Stretching mode of the Na^+-SM4s decreased from 1219 to 1215 cm^{-1} on the addition of

Ca^{2+} probably due to bridge formation between two SM4s head groups.[159] After permethylation, the dimethyl ester of sulfate exhibited a stronger and more discrete sulfate ester (-O-SO_2-O-) doublet between 1405 and 1198 cm^{-1}.[167,275] The R-O-SO_2-R group of SQ-A_2Gro or deoxyceramide sulfonate[12] shows an asymmetric $O{=}S{-}O^-$ stretch at 1160–1170 cm^{-1},[287,332,518] and symmetric $O{=}S{-}O^-$ stretch at 1030–1050 cm^{-1}.[159,272,332]. The $O{=}S{-}O^-$ of taurine amide was observed at 1096 cm^{-1}.[29,665] The relative size of $O{=}S{-}O^-$ stretching vibration to OH absorption (3450 and 1060 cm^{-1}) depicted the sulfate/monosaccharide ratio and was used to discriminate *bis*-sulfoglycolipids from mono-sulfo compounds[369,558,560] or SM4s from SM3.[539] The peak height at 961 cm^{-1} due to $SO_3{}^{18}O^{2-}$ was used to quantitate SO_4^{2-} released by fission of O—S bond.[482,601]

For a primary sulfate group on C-6 of a hexopyranose in the C1(**D**) conformation, a sharp but relatively weak absorption of the C-O-S stretching vibration appears at 810–820 cm^{-1}, while the primary C-O-S of HSO_3-PtdGro was observed at 840 cm^{-1}.[167] The sulfate at a secondary, equatorial position in the ring of a hexose,[217,568] hexosamine[149,560,565] and on C-8 of sialic acid[315] absorbs at 810–825 cm^{-1}, and the sulfate at a secondary, axial position at about 850 cm^{-1}.[369] Although the above absorption has been frequently referred to as diagnostic of the sulfate location on sugar, there are often ambiguities because the presence of different aglycon groups and substituents on a glycoside may profoundly alter the position of the absorption near 850 cm^{-1}.[186]

3. *Mass Spectrometry*

Because polar groups, especially sulfate, hinder the electron ionization (EI),[559] the ionization of native sulfatides was carried out by field desorption (FD),[170] fast-atom bombardment (FAB),[369] liquid secondary ion mass spectrometry (LSIMS), electrospray ionization (ESI),[215] or matrix-assisted laser desorption ionization (MALDI).[562,570] FAB and LSIMS spectra of underivatized sulfatides, in both negative and positive ion detection modes, may be able to supply information on the number and the location of sulfate(s) on monosaccharides.[215,324,437,566] Collision-induced dissociation (CID) and linked scan (or MS/MS) spectra contained product ions formed from a selected precursor (parent) ion or all precursors that give rise to a specific product (daughter) ion.[566]

(a) Electron ionization (EI)

Both molecule-related ions and carbohydrate sequence ions of sulfatides were obtained by EI–MS only after permethylation and desulfation.[235,561] The peaks of the saccharide linked to C1 and C2 of the sphingoid with a fatty acid plus one proton were most intense. The sulfate(s) was localized by solvolytic desulfation of the permethylated sulfatides followed by C^2H_3-remethylation of the hydroxyl that was originally occupied by the sulfate group.[561]

(b) Fast atom bombardment (FAB), liquid secondary ion mass spectrometry (LSIMS) and electrospray ionization (ESI)

Soft ionization methods including LSIMS[323,324,564–566] and FAB[215,437] produce a series of sequence ions in addition to the molecule-related ions. These ionization patterns are essentially similar to those of gangliosides[450] and unsaturated sulfated disaccharides obtained from glycosaminoglycans.[90] The amount of the sample for analysis can be reduced down to approx. 50–200 pmol and peracetylation or permethylation of sulfatides usually results in a better signal to noise ratio.[218,339,369]

Positive ion LSIMS of the potassium salt of SM4g and lyso-SM4g showed the molecular ions in various cation forms including $[M - H + 2Na]^+$, $[M - H + Na + K]^+$, and $[M + K]^+$ [324] because Na^+ is abundantly present in water, test tubes, pipettes and the probe. Addition of 0.5% NaCl to the matrix intensified $[M - H + 2Na]^+$ and $[M - 2H + 3Na]^+$ ion from mono- and *bis*-sulfoglycolipids respectively, as well as positive ions containing ceramides (Yn + Na), which can be used to delineate the monosac-

charide sequence.[562] The advantage of positive ion detection is the relative preponderance of ions arising from lipophilic residues including EAGro,[324] and sphingoids[450] although the sensitivity is lower than the negative ion detection. The survey of *bis*- and *tris*-sulfoglycolipids led to the general formula for the molecule-related positive ions: $[M - nH + (n + 1)Na]^+$ where n represents the number of charges[215] in analogy with mono- and disialosylganglioside GM1 and GD1a which also yield $[M + Na]^+$ and $[M - H + 2Na]^+$ respectively.[450]

FAB or LSIMS spectra of HSO_3-Chol,[40,384,464] SM4g,[10,324] HSO_3-3GalManGlcE_2Gro,[126,288] SMGM1a[567] as well as other monosulfated glycolipids[566] obtained in *negative* ion mode contained primarily the deprotonated molecule, $[M - H]^-$. The molecule-related ions of a potassium salt of SM4g,[324] an ammonium salt of monosulfated glycolipid, e.g. HSO_3-6ManGlc-E_2Gro,[383] or di-ammonium salts of *bis*-sulfated glycolipids, e.g. $(HSO_3)_2$-2,6ManGlc-E_2Gro,[369] were similar to those of the corresponding sodium salts. Glycerol-type sulfatides, e.g. SM4g, and the E_{20}Gro-containing trihexosyl sulfatide, yielded a peak due to 'lyso-SM4g', and $[M - E_{20}]^-$ respectively, with minimal intensities of fragment ions from lipophilic moieties. Instead, the major negative ion from SM4g and $(HSO_3)_2$-2,6ManGlc-E_2Gro was the ion consisting of the carbohydrate and glycerol.[562]. When applied to *bis*-sulfoglycolipids (SB2, SB1a and $(HSO_3)_2$-2,6Manα-2Glcα-1$E_{25}E_{20}$G ro), the molecule-related ions appeared in the form of sodium adduct ions, $[M + Na - 2H]^-$ accompanied by $[(M + Na - 2H) - NaSO_3 + H]^-$ ($[M - SO_3H]^-$) ions, which corresponds to the monodesulfated sulfolipid. SMUnLc$_4$Cer,[68] and SMGM1a,[567] which have two species of the negatively charged group, also produced $[M + Na - 2H]^-$ accompanied by ions formed by the loss of a sulfate, $[M - SO_3H]^-$. SMGM1a contained, in addition, $[M + Cs - 2H]^-$. The mass number of the molecule-related negative ions in polysulfoglycolipids was generalized in the formula: $[M + (n - 1)Na - nH]^-$.[215] Sugar sequence ions, including $[HSO_3$-Hex-*O*-HexNAc-*O*-Hex-*O*-Hex-*O*-Hex-(*O*)-$]^-$, and $[HSO_3$-*O*-Hex-*O*-HexNAc-*O*-Hex(-*O*-SO_3H)-*O*-Hex-(*O*)-$]^-$ respectively, were abundantly obtained by the normal scan spectra of SMiGb$_5$Cer[564] and SB1a.[324,566] The ions containing the sulfate and the parts of galactose $[SO_3OC(CH_2OH) = CHOH]^-$ (*m/z* 169) and $[SO_3OCH = CHOH]^-$ (*m/z* 139) were reported to arise from SM4s.[437]

The intense negative ion at *m/z* 97 (96.960) corresponded to the sulfate group plus hydrogen $[OSO_3H]^-$ (hydrogen sulfate anion) as confirmed by accurate mass measurement.[339] Both *m/z* 80 ($[SO_3]^-$) and 97 were well visible when distinct from the matrix peaks[323] and are the most convenient pair of ions to differentiate from a phosphate which yield the *m/z* 79/97 ($[PO_3]^-/[H_2PO_4]^-$) ion pair.

The single- and double-charged ions from synthetic mono-, *bis*-, and *tris*-sulfated Le^x-trisaccharide 1-propanols and from synthetic mono- and *bis*-sulfated sulfatides by ESI CID–MS/MS in positive[215] and negative modes[216] provided valuable information in identifying the sulfated sugar unit. Positive-mode ESI-mass spectrometry of a mixture of $CaCl_2$, GalCer and SM4s yielded $[GalCer\text{-}SM4s\text{-}Ca^{2+} - H]^+$ as the most stable noncovalent oligomer. These authors concluded that Ca^{2+} may mediate carbohydrate–carbohydrate interaction and might be involved in adhesion of the extracellular surfaces of the myelin sheath.[308]

(c) MALDI-TOF

Ions sublimated by matrix-assisted laser desorption ionization (MALDI) of lyso-SM4s[570] and sulfatides[562] were analyzed by time-of-flight (TOF) mass spectrometry both in positive and negative modes. MALDI spectra can be obtained with a sulfatide mixture containing only a 1/100 of the sample required for LSIMS and the negative ion profiles of molecule-related ions are similar to those obtained with LSIMS of the same glycolipids. Mono- and *bis*-sulfoglycolipids produced intense $[M - H]^-$ and $[M + Na - 2H]^-$ accompanied by $[M - SO_3H]^-$ ions respectively. The spectra obtained in the positive ion mode showed $[M - nH + (n + 1)Na]^+$ (where n = 1 or 2) as well as the fragment ions

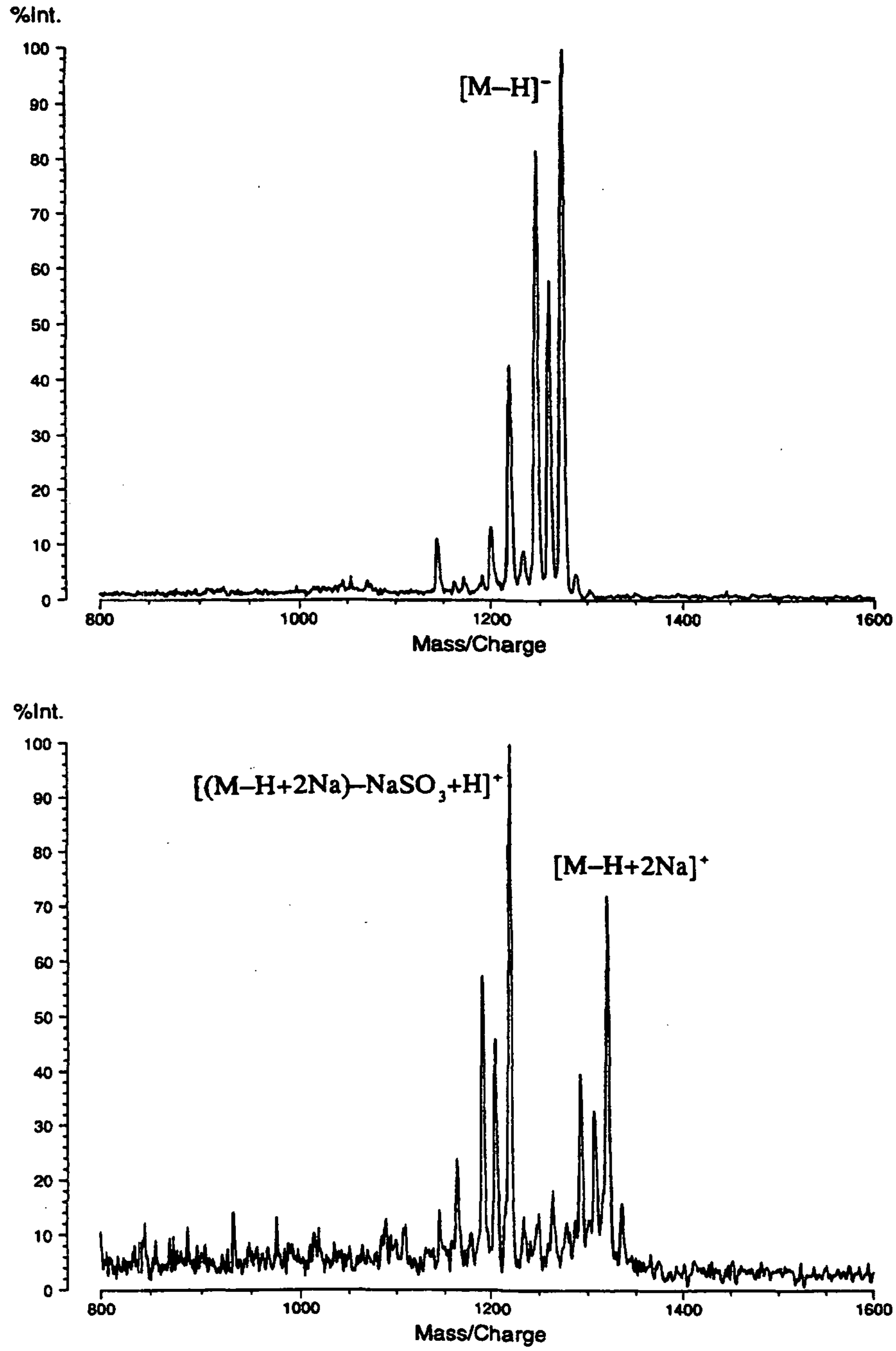


Fig. 4. MALDI–TOFMS spectra of SM2a from rat kidney in negative (upper panel) and positive ion modes (lower panel).

produced by the loss of one or two sulfate esters.[562] The initial setback of MALDI to produce only weak or no sequential ions is gradually improved by selection of novel matrices and by advanced hardware and ionization techniques including post source decay (PSD), (Fig. 4).

(d) Collision-Induced dissociation (CID)

The *high and low-energy* collision of the deprotonated molecule, i.e. $[M-H]^-$, produces sequence ions diagnostic for sulfatides.[126,215,288,437,565,567,568] These include oligosaccharide ions produced by the cleavage of the glycosidic bonds on either side of the anomeric oxygen, sequentially from the nonreducing terminus (e.g. $[(HSO_3$-*O*-Hex-(*O*)) − 2H]$^-$, [HSO_3-*O*-Hex-*O*-HexNAc-*O*-Hex(-*O*-SO_3H)-*O*-Hex-*O*-Hex-(*O*)-]$^-$),[565566] oligosaccharides linked to a lipophilic aglycon (e.g. [(*O*)-HexNAc-*O*-Hex(-*O*-SO_3H)-*O*-Hex-*O*Cer (t18:0/24:0)]$^-$,[568] [HSO_3-*O*-Hex-*O*-Hex-*O*-CH═$CHNH_2$]$^-$)[565] as well as the fragments produced by cleavage of the galactose (e.g. [$^-O_3SOC(CH_2OH)$═CHOH)[437] or glucose[215,565] ring. [M − fatty acid]$^-$ and [M − alkyl]$^-$ were the most intense and characteristic product ions from SM4g and lyso-SM4g.[562] Low-energy CID–MS/MS of both SM4s and SM4s-6 produced ions of m/z 169 or 167 [$^-O_3SOC(CH_2OH)$═CHOH or its dehydrogenated form] and 139 [$^-O_3SOCH$═CHOH], while the ions at m/z 119 and 151 were specific to SM4s and SM4s-6 respectively.[437] The CID–MS/MS of the $[M+Na]^+$ or $[M-H+2Na]^+$ also supplied abundant information on the sulfated sugar unit as well as the sequence.[215,562]

(e) Aglycons and molecular species

The representative negative ions containing ceramides (Yn ions) include [*O*Cer]$^-$,[449,564] [SO_3-Hex-*O*-CH═CH-NH_2]$^-$,[450] [*O*-Hex-*O*Cer]$^-$,[450,564] [*O*-Hex-*O*-Hex-*O*Cer]$^-$,[449,564] [*O*-Hex-*O*-Hex-*O*-Hex-*O*Cer]$^-$,[564] [*O*-HexNAc-*O*-Hex-*O*-Hex-*O*Cer]$^-$,[449] [(*O*)-Hex(-*O*-SO_3H)-*O*-Hex-*O*Cer]$^-$,[324,566] and [(*O*)-HexNAc-*O*-Hex(-*O*-SO_3H)-*O*-Hex-*O*Cer]$^-$.[566] Lyso-SM4s ([M − fatty acid]$^-$) was obtained only from SM4s with a 2-hydroxy fatty acid by FAB and low-energy CID in the negative mode, although the ion of the long-chain base was always detected by the positive ion mode.[437] Peculiar ions ascribed to the mechanism called charge remote fragmentation (CRF) occur in some cases. For instance the high-energy CID spectra of the $[M-H]^-$ ion contained a series of ions at high mass region, that were evenly spaced by 14 Da ($[M-H-16]^-$ and $[(M-H-16)-(CH_2)_n]^-$), as a result of C—C bond cleavage in the ceramide moiety by CRF.[565] The position of the double bond in 24:1 fatty acid was determined at C15 according to the intensities of CRF signals.[437] FAB analysis of SM4s from bovine brain and erythrocytes showed an $[M-H]^-$ corresponding to a molecular species with d18:1/24:1 at m/z 888 and d18:1/16h:0 at 794[323] respectively. Because other molecular species may yield incidentally $[M-H]^-$ at the same m/z, for instance with d18:1/22h:0 or t18:0/22:1 ceramide, comparison with $[M-H]^-$of acetyl or methyl derivatives was necessary to delineate the number of hydroxyls.[394]

Using a specially designed, motorized TLC–FAB–MS probe with continuous desorption and scanning over a moving TLC plate, glycolipids with identical polar heads were well resolved into molecular species with differences in long-chain base and fatty acid.[112,264] The technique was applicable to SM4s and SM3 isolated from the human kidney (IV.B). The molecule-related ions were successfully recorded by TLC–LSIMS with ordinary probe and down to 1 pmol of neoglycolipids were identified.[112] Recently a simpler method of TLC blotting was developed, where glycolipids are transferred to polyvinylidene difluoride (PVDF) membrane [cf. II.C.1(a)].

4. ^{1}H-NMR

Thanks to the increase in sensitivity requiring only 10 nmol and 1 μ mol of sulfatides respectively, ^{1}H- and ^{13}C-NMR spectroscopy developed into the method of choice for the structure determination of sulfatides. The monosaccharide composition, sequence, and linkage including anomeric configuration can be unequivocally determined by two dimensional ^{1}H homonuclear correlation spectroscopy (COSY),[564,567,568] homonuclear Hartmann–Hahn (HOHAHA), nuclear Overhauser effect (NOE) analysis,[568] and ^{1}H-^{13}C heteronuclear COSY.[369] The location of sulfates, and acyl esters[10,232] may be readily assigned by the chemical shift increment. The location of phosphate esters is determined,

in addition, by ^3J and ^4J ^{31}P- ^{1}H and ^{13}C-^{31}P couplings.[370] Also, the structure of aglycon including 4-hydroxysphinganine (t18:0)[565,568] and 1-alkyl-2-acylglycerol[232] is unequivocally determined. Moreover, some information on the conformation of molecule in solution was obtained (Iida-Tanaka, N. and Ishizuka, I. unpublished).

Most of the spectra since 1985 have been obtained in the solvent system of Dabrowski (deuterated dimethylsulfoxide/^2H_2O, 98:2) with deuterium-exchanged glycolipids at 60°C facilitating the direct comparison of chemical shift data.[82] The chemical shift and $^3J_{1,2}$ of anomeric protons can be measured with 10 nmol of sulfatides,[559] and with the material above 100 nmol, ^{1}H— ^{1}H coupling constants of H1–H6 of sugar ring protons obtained by interpretation of 2D spectra, help to distinguish the monosaccharide species.[9,131,369] Fortunately, the signals of the protons at α and β position to the sulfate ester (e.g. H6b, H6a, H5, and H2 of $(HSO_3)_2$-2,6Manα-2Glcα-1$E_{20}E_{20}$Gro) emerge out of the bulk signal region due to the large downfield shift.[369]

The H1 (axial), H2 (ax.), H3 (ax.) and H4 (equatorial) protons of the 3-sulfated β-galactose in SM4s (Iida-Tanaka, N. and Ishizuka, I., in preparation), SM4g, SM3,[347] SMiGb$_5$Cer,[564] and SMGM1a[565] resonated 0.09–0.12, 0.13–0.17, 0.64–0.71 and 0.29–0.37 ppm more downfield than those of the terminal galactose in GalCer, GalEAGro, LacCer, iGb$_5$Cer, and Gg$_4$Cer respectively. In contrast, the shift increments of the vicinal axial H2 of the internal 3-sulfated galactose of SM1a and SM2a were only 0.074 and 0.065 ppm respectively. The shift increments were largest with the α proton,[187] i.e. H3 of 3-sulfated glucose in SM4s-Glc (Δ0.835 ppm), and H2 (Δ0.70 ppm) of 2-sulfated mannose in $(HSO_3)_2$-2,6Manα-2Glcα-1E_2Gro.[369] The H1–H5, H6a and H6b of glucose 3-sulfate of SM4s-Glc (t18:0) resonated at more downfield by 0.122, 0.141, 0.835, 0.200, 0.080, 0, and –0.006 ppm respectively, in comparison to GlcCer (t18:0).[217] The smaller increment of the axial H4 of SM4s-Glc in comparison to the equatorial H4 of 3-sulfated galactose is in agreement with the results with synthetic oligosaccharides.[187] The chemical shift increment of H6a, H6b, and H5 of 6-sulfated GalCer (SM4s-6) was 0.300, 0.334, and 0.224 ppm respectively (Iida-Tanaka, N. and Ishizuka, I. unpublished), and H6a, H6b, and H5 of 6-sulfated mannose in HSO_3-6Manα-2Glcα-1E_2Gro was 0.395, 0.351, and 0.158 ppm.[369] In all the sulfated saccharides, the effect of sulfate esters on the coupling constant, $^3J_{H,H}$, and $^1J_{C,H}$ was minimal (Iida-Tanaka, N. and Ishizuka, I. In preparation).

The H1–H4 of the terminal 3-*O*-sulfated β-*N*-acetylgalactosamine in SM2b, SB2 and SMiGb$_4$Cer resonated at 0.01–0.05, 0.17–0.35, 0.39–0.59 and 0.45–0.47 ppm more downfield respectively, as compared with those of Gg$_3$Cer, SM2a, and iGb$_4$Cer respectively.[565] The shift increment of H4 ring protons was significantly larger than those of vicinal H4s of the 3-*O*-sulfated galactose, probably due to the acetamide group. The law of additivity was applicable to the chemical shift increment of the protons of 3-sulfated GalNAc in SB2, and SM2b, vs. SM2a and Gg$_3$Cer. The shift increment of GalNAc-H3 in SM2b (0.579 ppm) added to that in SM2a (–0.146 ppm) made 0.433 ppm, which was roughly consistent with the increment of GalNAc-H3 in SB2 (0.472 ppm).[565] Similar relationships were observed among GM1a(NeuGc) IV^3 sulfate, GM1a(NeuGc), and Gg$_4$Cer.[567]

A hydroxyl or an electron-rich group in the aglycon which is spatially close to the ring proton but separated more than three bonds shifts the saccharide proton signals of sulfatides downfield.[337,564] The shift increment of H1 of hexose by the hydroxyl at C4′ of t18:0 and the 2-hydroxy group on the amide-linked fatty acid was approximately 0.02 ppm (Iida-Tanaka, N. and Ishizuka, I. In preparation). Inversely, when the amide on C2′ of sphingenine was deacylated as in lyso-SM4s, chemical shift increments to H1′ to H5′ of d18:1, especially to the signals of H1′, H3′ and H5′ were substantial.[569] The dimethyl ester of HSO_3-PtdGro[166] and permethylated 6-sulfo-Gal-ManGlc-E_2Gro from *H. salinarum* (*cutirubrum*)[275] resonated at 3.98 ppm showed a sharp singlet at 4.0 ppm (three protons) attributable to the secondary S-*O*-CH_3 group (Fig. 5).

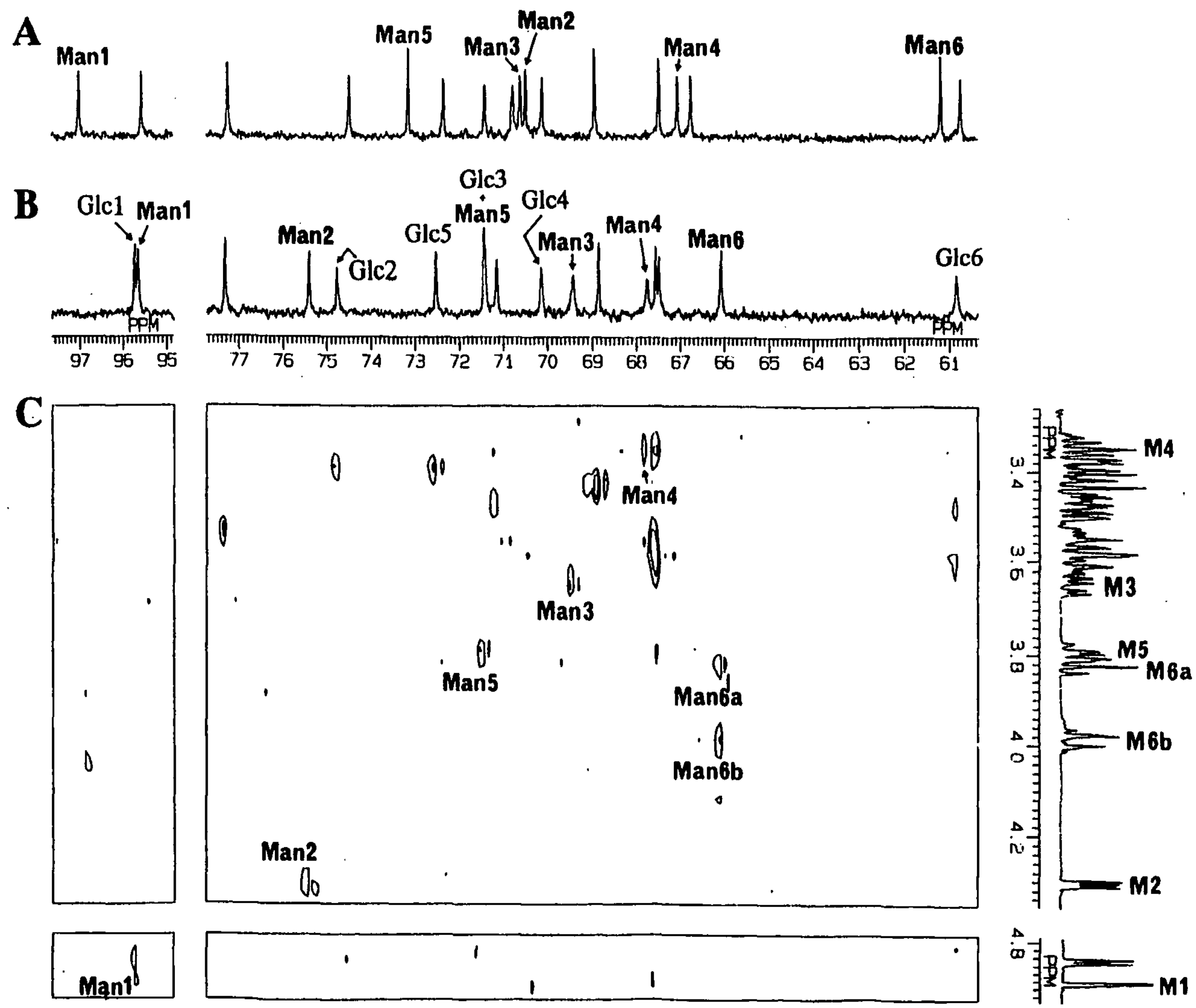


Fig. 5. Two dimensional H-C COSY spectrum (C) of $(HSO_3)_2$-2,6Manα-2Glcα-1$E_{20}E_{25}$Gro from halophilic bacterium *Natrialba asiatica*. (A) and (B), the one dimensional carbon spectrum of this glycolipid and its desulo-derivative respectively.

5. ^{13}C-NMR

One μ mol of sulfatides is sufficient to obtain ^{13}C-NMR spectra either in one dimensional (1D) proton-decoupled or distortionless enhancement by polarization transfer (DEPT) mode, while several μ mol of sulfatides are necessary to assign ^{13}C signals by relating to ^{1}H signals using two dimensional (2D) heteronuclear analyses such as ^{1}H-^{13}C COSY,[369] or ^{13}C-^{1}H HMQC. The shift increment of the saccharide carbons by the sulfate ester was essentially similar to those with sulfated oligosaccharides.[187, 322] Downfield shifts of approx. 6 ppm for C-3 due to increased electronegativities accompanied by approx. 1.5 ppm of upfield shifts for the vicinal carbons at β-positions (C-2, C-4) were observed for the 3-sulfated galactose of SM4s, SM4g, SM3 (Iida-Tanaka, N. and Isizuka, I. In preparation), and lyso-SM4s[569] as well as 2,6-*bis*-sulfated mannose of the sulfatide from a halotolerant archaea.[369] ^{13}C chemical shifts are sensitive to the total environment of the particular carbon atom and may be affected by changes in molecular structure six or more atomic centers away[156] including 2-hydroxyl group of the fatty acid in ceramide. The downfield shift of protons at C-1, -3, and -5 of d18:1 in lyso-SM4s relative to GalSph indicated that the removal of the sulfate from SM4s might increase the degree of rotational freedom at C-2–C-5 of d18:1, thereby inducing the zigzag conformation of d18:1 in lyso-SM4s.[569]

IV. STRUCTURE

Variations in both hydrophilic and lipophilic residues contribute to the divergence of sulfatide structures in the biosphere.[238] The variety of oligosaccharides may serve as the spacer at the cell surface.[491] Representative naturally occurring sulfatides including SM4g,[141] and HSO_3-6E_2Gro[257] were totally synthesized.

A. Sulfated Saccharide

The component sulfated monosaccharide in vertebrate glycolipids is exclusively the pyranosides (*p*) of β-Gal, β-Glc, β-GalNAc, and β-GlcU sulfated at the equatorial C3 hydroxyl. The saccharide structures of the major animal sulfatides, SM4s, SM3, SM2a, SB1a, and SMGb$_5$Cer are similar to the corresponding sialic acid analogues, gangliosides, GM4, GM3, GM2, GD1a, and V^3-NeuAc-Gb$_5$Cer respectively.[233] The sulfatides of echinoderms contain NeuGc sulfated at C8; archaeal sulfatides, α-Man*p* sulfated at C2 and/or C6 hydroxyl, β-3-sulfated Gal*p*, or *sn*-1-sulfated Gro; and mycobacterial sulfatides, 2-sulfated α-Glc*p*. Rat kidneys[560, 561] and an extremely halophilic archaea *Natrialba asiatica*[369] contain *bis*-sulfated glycolipids that possess two sulfate groups attached to the separate monosaccharides and to the identical monosaccharide respectively. The 2-sulfate on mannose of a halophilic archaea is the only example of the axial glycolipid sulfate.

The sulfated saccharide interacts tightly with water molecules in the aqueous system. Water layers of thickness up to 44 Å, formed between the polar head groups in the gel state, were stable for several days.[6] It has been known that proteoglycans are inflated with water to form gel with high internal osmotic pressure and thus contribute osmotically and, on the other hand, hamper the transport of ions. Hence the surface mucous exerts cellular osmoregulation by means of altering the ion gradients at the cell surface.[78] Similarly, sulfatides[238, 426] and HSO_3-Chol[418] may act as one of the ion barriers or ion traps on membranes and protect the cell against high extracellular osmolality in tight cooperation with the cell surface 'barrier mucus' (cf. IV. A), which provides the Donnan equilibrium.[76, 192] The sulfated glycosaminoglycan concentration and composition in 15 species of Crustacea, Pelecypoda and Gastropoda living in different degrees of salinity demonstrated a direct correlation between the logarithm of the sulfated glycosaminoglycans and the salinity for all the species analyzed.[146]

B. Lipophilic Residue

The structure of lipophilic residues of glycolipids in eucaryotic cells varies considerably depending on the polar head groups[525] and in turn the molecular species of sulfoglycolipids with identical polar head groups vary with their specific tissue location.[238] This diversity of lipophilic aglycons is essentially determined genetically but susceptible to the developmental stages and alteration of environments.[45] In contrast, the variation of the lipophilic residue concomitant with elongation or modification of carbohydrate chain is not prominent in archaea[273] and eubacteria.[117,238] For instance, in gram-positive cocci, fatty acid compositions of glycolipids are only slightly distinct from those of phospholipids.[117] Although cellular accumulation of diacylglycerols, alkylacylglycerols, ceramides, long chain bases or sphingosine 1-phosphate might transduce hormonal signals, and regulate growth and apoptosis, the relation to sulfoglycolipid has never been recognized.

1. Acyl-, Alkylglycerols and Ceramides

Diacylglycerols (A_2Gro): The aglycon of the major plant anionic glycolipid containing 6-sulfoquinovose is exclusively A_2Gro[188] (Fig. 1). The concentration of SM4g (1.69 μmol/g) exceeds that of SM4s (1.35 μmol/g) in the brain of Alaskan pollack indicating the dominance of glycerol-type sulfatides.[577] SM4g in the brain of gadoid (cod) fishes contains mainly (> 90%) A_2Gro with 18:1 and 16:0 fatty acids.

Alkylacyl- and dialkylglycerols (EAGro and E_2Gro): Alkylacyl- and diacylglycerols can be determined separately by TLC[466,530] or gas chromatography.[228,396] The mammalian testicular sulfatide (SM4g) contains almost exclusively a 1-alkyl-2-acyl-*sn*-glycerol[232] ($E_{16:0}A_{16:0}$Gro)[606,631] (Fig. 1). This simple and saturated nature of the lipophilic residue is in striking contrast to testicular phospholipids. SM4g in aged humans contained alcohols and fatty acids of slightly longer chain than those in younger men.[606] In HSO_3-3Gal-EAGro of the brain of rat[343] and gadoid fishes,[577] 16:1, 16:0 and 18:1 alcohols predominated. Vertebrate myelin SM4g contains varying proportions of EAGro.[238] The relative amount of EAGro and A_2Gro forms of SM4g in the brain of rat at the peak of myelination (21 day) was about 1:1.[227,452] The A_2Gro form of SM4g diminished more rapidly than the EAGro form[452] so that the ratio of EAGro to A_2Gro form at 175 days was about 13:1[227] (V.C.2(a)). Diphytanylglycerylether ($E_{20}E_{20}$Gro) and its dimer, dibiphytanyl-diglyceroltetraether are the components of the membrane archaea.[273] The genera Halococcus and Natrialba contain both $E_{20}E_{20}$Gro and $E_{25}E_{20}$Gro.[256]

Ceramides: Ceramides are distributed in plant, and fungi including yeast, and appeared in the animal kingdom from as early as sponge, the most primitive multicellular animal.[191] The molecular species of sulfatides from rat kidneys were clearly depicted in LSIMS[368,566,567] and MALDI–TOF spectra (Fig. 4). The presence of hydroxyl groups on C-2 of the fatty acid and C-4 of sphinganine enables sulfatides to form extensive hydrogen bonds on epithelial cell surface and thus strengthen the membrane[264,359,657] and may also influence the transcellular influx of water.[158] It is notable that t18:0/24:0 predominated in SM4s-Glc from rat kidneys, whereas the major ceramide of the putative precursor GlcCer was t18:0/22 h:0.[217] Similarly, glycolipids in the intestinal cells of rat and man possess ceramides consisting of t18:0/2-hydroxy fatty acid.[173] Recently it was reported that the ceramides with 2-hydroxy fatty acids were selected as the substrate for galactosyltransferase at the endoplasmic reticulum,[510] and glycosphingolipids with hydroxy fatty acids including GalCer and SM4s were preferentially sorted to the basolateral membrane at the trans-Golgi network of MDCK cells[657] shedding light on the regulatory mechanisms of the ceramide species.

2. Fatty Acids, Alcohols and Long-chain Bases

Saturated and monounsaturated fatty acids: The acyl component consisted predominantly of 16:0 (58%) in SQ-A_2Gro of the marine green alga, *Enteromorpha flexuosa*[518]

and some green algae contained exclusively 16:0 acid in lyso-SQ-A_2Gro.[133] Whether the major fatty acid (14:0) of SQ-A_2Gro in sea urchin[287] comes from algae or not is yet to be settled (V.B).

Mammalian testicular and sperm SM4g,[232] rat brain SM4g[343] and porcine pancreatic SM4s[407] contained 100, 80, and 60% respectively, of relatively shorter chain (16:0 and 18:0) saturated fatty acids. On the contrary, the predominant fatty acids of SM4s in the myelin of mammalian central nervous system were C24:1, and 2-hydroxy saturated.[618] SMUnLc$_4$Cer from mammalian central nervous system contained mainly relatively shorter chain fatty acids, 16:0, 18:0, and 18:1, amounting to 85% of the total fatty acids, whereas SMUnLc$_4$Cer from peripheral nervous system contained a large proportion (59%) of long-chain fatty acids ($>$18:0).[67] Mammalian kidney sulfatides were rich in saturated 22:0, 23:0 and 24:0 acids[401,561] (Fig. 4) and in particular, C22:0 which is found in quantities more than 10 times as much as those in the brain.[302] SM4s and SM3 of human liver,[539] and SM4s of rabbit serum[653] also contained 22:0 acid in proportions comparable to 24:0.

2-Hydroxy fatty acids: The ceramide of HSO_3-8NeuGc2-6Glc$p\beta$-1Cer from sea urchin consisted of 4-hydroxysphinganine (t18:0), and 2-hydroxylated acids (22 h:1, 23 h:1, 24 h:1).[315] The typical ceramides of vertebrate brain SM4s were d18:1/24:1 and d18:1/24h:1.[577] The content of 2-hydroxy acids in GalCer and SM4s was lowest in the brain of hatching chicken, shark and tuna.[638] Substantially lower concentrations of hydroxy fatty acids were also found in the brains of cartilaginous deep-water fish compared with surface fish,[344] and SM4s from Alaskan pollack and other gadoid fishes lacked in hydroxy fatty acids.[577] Both GalCer[247] and SM4s[1,302] of mammalian brain contained high proportions of long chain fatty acids with a 2-hydroxy group in D-configuration.[448] Nearly all of the 2-hydroxy fatty acids found in brain lipids were the constituents of these two glycolipids. In the GalCer-deficient animals, however, the ceramide with 2-hydroxy fatty acids was used for the synthesis of GlcCer and sphingomyelin.[75] During the first postnatal month of Wistar rats, the ratio of hydroxy- over non-hydroxy-species (HFA/NFA) of cerebral GalCer increased to 2.0, whereas the HFA/NFA ratio for cerebral SM4s declined to 0.6 in the same period.[88,431] The developmental change of 2-hydroxylation in the nervous system may be regulated by thyroid hormones.[578]

SM4s and SM3 of the extraneural organs including human liver,[539] mammalian kidneys,[398,559] and rabbit serum[653] were also characteristic for high contents of 2-hydroxy fatty acids. The contents of SM4s with 2-hydroxy fatty acids in the total SM4s in the kidney of human,[362] porcine intestine,[548] and rabbit fundic mucosa[413] have been reported to be 89, 67 and 80% respectively, whereas SM3 of the kidney of human[264] and house musk shrew contained only 0 and 25%[398] respectively. A similar fatty acid pattern was found in human urine SM4s, which probably originated from the kidney.[302,609]

Polyunsaturated and other fatty acids: The major fatty acids in the chloroplast SQ-A_2Gro were *trans*-hexadecenoic and 18:3.[63,188,284] The major characteristic fatty acids of the sulfonolipid isolated from a marine diatom *Nitzschia alba* were 16:1-Δ^3-*trans* for deoxyceramide sulfonate, and 20:5 for SQ-A_2Gro.[12] SQ-A_2Gro from the thermoacidophilic rod *Sulfolobus acidocaldarius* was unique in containing 17br, 17cyc, and 19cyc (cyc designates 11-cyclohexylundecanoic and 13-cyclohexyltridecanoic).[332] It has been reported that unsaturated fatty acids were abundantly present in the brain ganglioside of the stenothermic cold-water fish species,[21] while no systematic studies have been performed on the lipophilic residues of sulfatides and the environmental temperature.

Long-chain alcohols: The synthetic sulfated oligosaccharides that have long alkyl groups (C12–C18) at the reducing end exhibited tens to hundreds times higher anti-HIV activities than those of the corresponding sulfated oligosaccharides without alkyl groups, probably due to the detergent effects.[277]

Long-chain bases: The predominant long-chain base of SM4s in the vertebrate nervous system is sphingenine (d18:1). For instance the long-chain bases of SM4s from the brain of Alaskan pollack and other gadoid fishes consisted exclusively of d18:1.[577] The long-chain bases of SM4s from the salt gland of dogfish[269] and the equine kidney[181,570] were

unique in containing comparable amounts of dihydroxy and trihydroxy long-chain bases with 16–20 carbons. Bases with methyl branches at position 2, 3 or 4 from the methyl end were relatively abundant in shark brain SM4s.[269] Sphinganine (d18:0) predominated in the long chain base fraction of SM4s from the brain of snake, tadpole, and frog.[485,578]

The proportion of t18:0 was approx. 8% in SM4s from the whole human kidney, and SM4s and GM4 from rat kidney.[559] Interestingly, the proportion of t18:0 was 8% in the cortex and 34% in the medulla of human kidneys, and 11% in the cortex and 27% in the medulla of bovine kidneys, forming a concentration gradient from the cortex to medulla.[491] On the other hand, more than 70% of the long chain base was t18:0 in SM2a, SM2b, and other rat kidney sulfatides with oligosaccharide chain longer than three monosaccharides.[565]

C. Chemical Modification

1. Sulfate

Partial (limited) acid or alkali catalyzed hydrolysis of the sulfate ester of sulfatides aims to remove the sulfate ester at a mild condition without destroying the remaining part of the molecule,[456,603] while partial hydrolysis with stronger acid may provide sequence information. Solvolysis in dioxane or pyridine and acid catalyzed desulfation developed for steroid sulfates, are also effective especially for less polar sulfolipids. The discrimination of acid-catalyzed 'hydrolysis' and 'solvolysis' is marginal and both terms have often been used synonymously. Enzymatic release of inorganic sulfates was successfully applied to urinary metabolites of steroids and bile salts.[482] Invertebrate sulfatases[645] or partially purified human placental arylsulfatase A[165] released approx. 70% of the sulfate esters from SM4s. However, there has been no broad specificity sulfatase available, which can quantitatively cleave the sulfate esters of sulfatides.[456] 6-Sulfoquinovose was released from the lipophilic group by incubation with β-galactosidase from *E. coli.*[281]

The sulfate ester of sulfoamphiphiles is easily solvolyzed in various organic, oxonium ion-forming solvents.[159] The HSO_3-Chol fraction was solvolyzed[580] or 'hydrolyzed' in acidified organic solvents.[243] The sulfate esters of more simple sulfatides including SM4g, SM4s or HSO_3-PtdGro can be quantitatively removed by solvolysis in dioxane at 100°C for 10 min[159,228,232,318] when water is not present.[167] Even sulfatides with two or three monosaccharides were completely desulfated: SM2a (methanolic dimethylsulfoxide containing 4.5 mM H_2SO_4 at 80°C for 3 hr),[559] HSO_3-ManGlcE$_2$Gro (dioxane/pyridine (1:1, v/v) at 100°C for 2 hr),[597,598] HSO_3-GalManGlcE$_2$Gro (4 mM HCl in anhydrous tetrahydrofurane at ambient temperature for 90 min,[273] and the ganglioside containing 8-*O*-sulfated NeuGc (dioxane or pyridine/dioxane at the same temperature.[297,315] The cleavage of the sulfate ester was also complete in 4–5 hr when the Kantor and Schubert method of mild desulfation for chondroitin sulfates in 0.05 M methanolic HCl is applied to SM4s,[362,415] SM3,[535] SM4g,[228,232] or HSO_3-3Galβp-6(Galfα-3)Manpα-2Glcpα-1E$_{20}$E$_{20}$Gro.[527] Analogously, SMUnLc$_6$Cer was desulfated[68] in 16 hr with 0.1 N methanolic HCl.[221]

On the other hand, solvolysis in dioxane or pyridine/HCl is often inadequate for sulfatides with more than two monosaccharides (e.g. SM1b, SMiGb$_5$Cer) or with the sulfate ester at the internal monosaccharide (e.g. SM2a).[561] Incubation with 5 mM HCl in dimethylsulfoxide containing 0.5% methanol at 80°C for 1–2 hr quantitatively converted SM2b,[565] SMiGb$_4$Cer,[564] and SMGM1a[567] into Gg$_3$Cer, iGb$_4$Cer, and GM1a respectively, whereas SM2a with an internal sulfate ester was unchanged after 1 hr.[565] After acid-catalyzed solvolysis of SB2 in 8 mM H_2SO_4 in 10% methanol in dimethylsulfoxide at 80°C, most of SB2 was converted into the monosulfo-analogue (SM2a) in 10 min, confirming that the sulfate ester on the terminal monosaccharide was much more labile to solvolysis,[560] whereas the internal sulfate ester of SM2a was resistant.[565] These obser-

vations suggested that the sulfate at C3 of the internal Gal is protected against acid by the GalNAc residue at C4 similarly to the internal sialic acid of GM1 ganglioside.[229]

Sulfate esters are stable under mild alkaline conditions used for deacylation.[361, 362, 535, 570] When 6-sulfates of Glc, Gal and Man were treated at higher temperatures (e.g. 0.1 M NaOH, 100°C) 3,6-anhydrohexosides were produced provided that C-3 has a free hydroxyl group.[159, 603] Conversely, alkali-treatment of SM4s, which contain a 3-*O*-sulfate and a free C6-hydroxyl, yielded a substantial amount of 3,6-anhydro-GalSph with the anhydrogalactose in the boat conformation.[432]

2. *Limited Acid Hydrolysis and Periodate Oxidation*

More than 60% of the sialic acid was released from GM3 in 0.1 M HCl at 37°C, whereas only 10% of the sulfate ester of SM4s was cleaved suggesting that the terminal sulfate ester was more resistant than the terminal sialic acid.[413] However, 5 mM HCl in dimethylsulfoxide containing 0.5% methanol cleaved preponderently sulfate from SMGM1a to yield GM1a, whereas the treatment of the same sulfated ganglioside with 10 mM formic acid[229] selectively cleaved sialic acid and yielded SM1b confirming that the effect of solvolysis is specific to sulfate esters.[567] Also oligosaccharides with 8-*O*-sulfated *N*-acetylneuraminic acid were released from sea urchin sulfated gangliosides by mild acid treatment using 0.1 N trifluoroacetic acid.[218] The ester migration under acid conditions, akin to that observed with certain sugar phosphates, does not occur readily with sugar sulfates.[603] Mild acid hydrolysis in the chloroform/methanol/water or 0.25 M methanolic HCl system was successfully applied to the identification of desulfated and partially hydrolyzed products from $(HSO_3)_2$-ManGlcE$_2$Gro,[369] and HSO_3-GalManGlcE$_2$Gro[275] respectively. 3-Sulfated galactose in SM4s and SM3[366] was not attacked by periodate.[228, 362, 637] The glucose at the nonreducing end was also resistant to periodate because of inaccessibility of the reagent to glucose due to micelle formation.

3. *Lipophilic Residues*

Stronger alkali and well-controlled conditions are necessary to chemically deacylated SM4s because the amide-linked fatty acid of ceramide is resistant to mild alkali and the yield is only 30–60%.[125, 307, 432, 569] Recently, a rapid method was developed to obtain lyso-sphingolipids in only 2 min using a microwave oven.[570] Also a novel enzyme that hydrolyzes the *N*-acyl linkage between fatty acids and sphingosine bases in ceramides of various sphingolipids was purified from the culture filtrate of *Pseudomonas* sp. TK4, which released 59% of fatty acid from SM4s at the optimum condition.[241] The acyl ester on the glycerol of SM4g was released by a lipase activity of rat liver lysosome.[465]

The hydrazine-nitrous acid fragmentation procedure provided valuable information regarding the extent and position of sulfation on the various carbohydrate units. The ^3H-labeled HSO_3-3AnTalH$_2$ [AnTalH$_2$ = 2,5-anhydro-D-talitol] was prepared from SB2 by hydrazine-nitrous acid treatment followed by NaB^3H_4 reduction.[98] Similar treatment of bovine lutropin yielded HSO_3-4AnManH$_2$ and HSO_3-4AnTalH$_2$. The diacylglycerol residue can be released from SQ-A$_2$Gro by periodic acid oxidation in methanol and fission of the glycosidic linkage with 1,1-dimethylhydrazine.[193]

4. *Methylation*

Acetolysis followed by reduction yields partially methylated alditols, are then separated by TLC[230] or gaschromatography after acetylation,[561] providing clues to the position of the sulfate ester and glycoside attachment.[565] Treatment with methylsulfinyl carbanion[13, 74] leads to deacylation of acyl esters, dephosphorylation, and *N*-methylation of fatty acid amide including ceramide,[564, 568] whereas desulfation during the process was minimal.[559, 564, 568] By the use of NaOH instead of NaH,[74] the permethylated glycolipids recovered from the lower phase of Folch's partition showed homogeneous bands on

TLC without by-products or contaminants, indicating that further purification was not necessary.[564] For methylation of the sulfate and phosphate esters of HSO_3-PtdGro, the free acid form of the sulfolipid was treated with ethereal diazomethane. The dimethyl ester was unstable in solution and decomposed within several hours to diphytanyl glycerol ether.[167]

V. FUNCTIONAL DISTRIBUTION

Sulfatides are distributed in three widely separated phyla, halophilic archaea,[256] Mycobacteria[149] and animals of the deuterostome lineage from echinoderms to vertebrates.[238] On the other hand, the sulfonoglycolipid, SQ-A_2Gro, is distributed in cyanobacteria, green and brown algae and thylakoid membrane of chloroplasts (cyanobacterial symbiotes) of higher plants.[63,498] Table 3 summarizes the structure and cellular concentration of sulfatides and related lipids classified according to the phylogenetic tree and the tissue of animals.

A. Prokaryotes and Plants

Extreme halophiles (Family *Halobacteriaceae*), which can grow in media containing up to 4 M of NaCl, belong to the distinct taxon Archaea (formerly Archaebacteria) of the kingdom *Prokaryotae*.[256] Sulfatides of halophilic archaea are useful taxonomic markers. Genera *Natrialba*, *Halorubrobacterium* and *Halobacterium* contain $(HSO_3)_2$-2,6Manα-2Glcα-1$E_{20}E_{20}$Gro (and $E_{25}E_{20}$Gro);[369] HSO_3-2Manα-4Glcα-1$E_{20}E_{20}$Gro; and HSO_3-3Galpβ-6Manpα-2Glcpα1-1$E_{20}E_{20}$Gro + HSO_3-3Galpβ-6(Galfα-3)Manpα-2Glcpα-1$E_{20}E_{20}$Gro respectively.[256] *Halococcus* and*Haloferax* contain HSO_3-6Manpα-2Glcpα-1$E_{20}E_{20}$Gro, while HSO_3-PtdGro ($E_{20}E_{20}$ analogue) is present in *Haloarcula*, *Halobacterium*, and *Halorubrobacterium*.[100] *Haloferax mediterranei* maintains a value (1.3–1.6), for the number of negative charges per mol ionic lipid, which is comparable to that (1.7-2.4) for *H. salinarum* and for *Haloarcula marismortui* (1.7–2.3).[326] With the growth of *Haloferax mediterranei* in media of increasing salt concentrations (3–7M), gradual increase in the relative proportion of the sulfated diglycosyldiether (S-DGD-1) (21–37 mol%) was observed. In addition, the proportion of the diphytanylether analogue of HSO_3-PtdGro was 1/3 as great in 7M salt as in 3M salt.[325] A halotolerant strain of *Staphylococcus epidermidis* also responded to high NaCl concentrations (up to 4M) in the growth medium with an increase in the percentage of 6-glycerophosphoryl-β-gentiobiosy-A_2Gro and cardiolipin.[303] As a result, the average number of negative charge/mol phospholipid increased from 1.01 to 1.14. All data above supported that these anionic lipids play a role in controlling the ion permeability of the cytoplasmic membrane similarly to sulfatides of the plasma membrane of animal transporting tissues.[267] Sulfoamphiphiles at the outer leaflet of the cell membrane appear to cooperate with the highly acidic surface layer glycoprotein as ion-barriers[376] (cf. IV.A; V.C.1).

Sulfatides of *M. tuberculosis* were discovered in the search for a strain reactive to weakly basic phenazine dye, neutral red, located at or near the surface of virulent human and bovine strains of the pathogen.[149] Mycobacterial 'sulfolipids' contain the monosulfated α,α-trehalose core acylated with complex branched-chain fatty acids.[19] Mycobacterial sulfatides elicit activities of neutrophils[635] (V.C.3), although the possibility to be the major determinant of virulence in tuberculosis seems unlikely.[149]

A nonphotosynthetic marine diatom *Nitzschia alba*, a eukaryote, contains sulfonoceramides (d18:1) and HSO_3-24-methylene-Chol.[12] Capnine (1-deoxy-15-methylhexadecasphinganine-1-sulfonic acid) and its acylated derivatives are the major components of the cell envelope of gliding bacteria.[145] The plant lacks sulfatides. Instead, photosynthetic microorganisms including cyanobacteria, algae, photosynthetic diatoms, and higher plants contain a sulfonoglycolipid (SQ-A_2Gro) accounting for as much as 40% of the total lipids.[274] In eukaryotic photosynthetic organelles SQ-A_2Gro is located at the thylakoid membrane of chloroplasts,[155,159] predominantly in the inner leaflet of the bilayer.

SQ-A_2Gro was localized in the photosystem II, suggesting that SQ-A_2Gro may be responsible for PSII activity by associating with the core and light-harvesting complexes of PSII.[497]

The SQ-A_2Gro deficient mutants of a photosynthetic purple bacterium *Rhodobacter sphaeroides*, and the cyanobacterium Synecoccus sp. did not have apparent phenotype including the photosynthetic electron transport system.[155] The growth of the wild type of these organisms under phosphate limitation resulted in increased amounts of SQ-A_2Gro with concomitant decrease of phosphoglycerolipids.[31] By contrast, the SQ-A_2Gro-deficient mutant maintained a normal level of phosphatidylglycerol.[155] These authors concluded that SQ-A_2Gro plays no significant role in photoheterotrophic growth or photosynthetic electron transport in *R. sphaeroides* but may function as a surrogate for phospholipids, particularly phosphatidylglycerol, under phosphate-limiting conditions. Analogous examples of replacement of phosphate of lipids and polysaccharides with carboxylic acid residues have been reported in eubacteria.[238] In contrast, another mutant of photosynthetic microorganism lacking in SQ-A_2Gro, with concomitant increase of phosphatidylglycerol showed alterations in photosynthetic activity.[497]

B. Invertebrates, Fishes, Amphibia, Reptiles and Birds

The protostome lineage, including Molluscs and Arthropods, lacks sulfatides[238] and sialic acids,[501] instead contains lipids with other anionic or zwitterionic groups including uronic acid, aminoethylphosphonate,[212] and phosphate esters of carbohydrates in insects.[628] Sulfatides have been reported only from Deuterostomia (Echinodermata and Chordata).[238,441] Echinoderms contain HSO_3-Chol, SM4s and/or gangliosides with sulfated *N*-glycolylneuraminic acid (NeuGc).[296] Although the true metabolic origin of SQ-A_2Gro in the sea urchin is as yet unclear, it is interesting from a food-chain point of view that both the green alga and the sea urchin inhabit the same sea areas.[287]

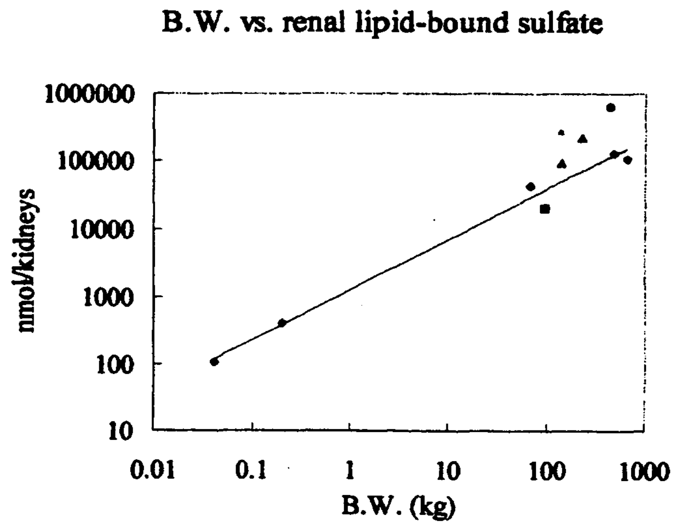
Eptaretus burgeri (a cyclostome) is one of the most primitive vertebrates (the Chordate branch of Deuterostomia), and its myelin does not contain detectable amounts of glycolipids.[575] The myelin structures are first apparent in cartilagenous fish species.[441,575,578] Elasmobranchs, sharks and rays, may stand closer to higher vertebrates than to the Teleosts with their high concentrations of brain glycolipids including SM4s.[311,441] The nerve membranes of Gadoid fishes (Alaskan pollack and Pacific cod) contained SM4g (seminolipid).[228,576,577] In embryos of sweet water fish, medaka (teleost), SM4s was detected throughout the skin and alimentary canal by staining with a monoclonal antibody.[113] SM3 was present in fish and avian testes[342] and was the major glycolipid of salmon eggs,[347] whereas SM4s was detected in the testes of puffer (Pisces),[606] turtle (Reptilia), and bullfrog (Amphibia).[342] Osmoregulatory organs of vertebrates including the gills of eel,[654] the rectal gland of shark, and the salt gland of sea gull and duck[267] contain SM4s (cf. V.C.1). Adaptation of eider duck to saline increased the concentration of SM4s in the salt gland dramatically suggesting a role for SM4s in NaCl excretion.[270] On the other hand, the turnover of SM4s of the gills of eels adapted to seawater for six weeks was greatly enhanced, although the tissue concentration was not significantly different from that of eels adapted to fresh water.[654] The skin and gills of tadpoles from a Chilean frog contained appreciable concentrations of SM4s that increased with differentiation.[147] SM4s also occupied about 87% of the total acidic glycolipids of *Xenopus laevis* oocytes.[664] In oocytes and embryos of *X. laevis*, SM4s was distributed in the cytoplasm of vegetal hemisphere, and in neurula stage, in endoderm.[316] The concentration of SM4s in brain of *X. laevis*[442] and bullfrog tadpole[578] changed quantitatively during their metamorphic stages.

The concentration of SM4s in chicken retina increased with age whereas SM4g decreased similarly to rat brain resulting in the contents of SM4g in sulfatides of 19.6%

in 18-day-embryo and 6.7% in adult (> 70 days after hatching).[95] The highest activity of GalCer sulfotransferase and arylsulfatase A in the brain and retina of chicken was observed just before hatching.[95] SM4s was the major glycosphingolipid of mature duck testes.[342]

C. Mammals

Mammalian epithelial tissues contain varieties of sulfatides, whose structure and concentration can be determined genetically and developmentally. Sulfatides are expressed at the outer leaflet of the plasma membrane of the glandular epithelial cells.[361]



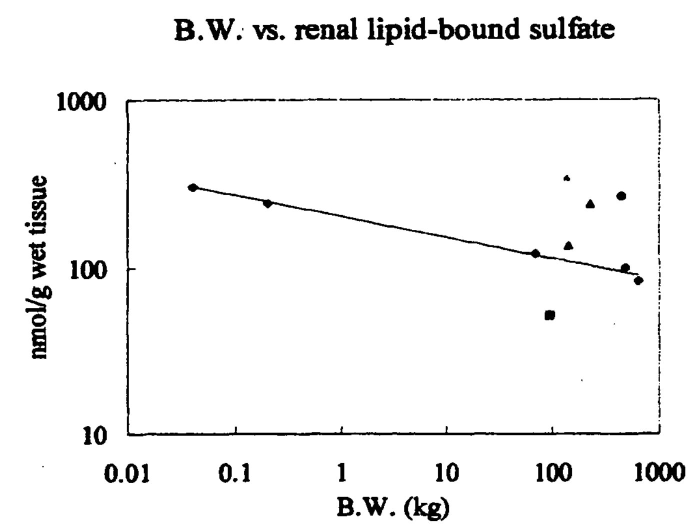


Fig. 6. Allometric relation between the renal concentration of the sulfatide sulfate and the body mass of mammals. ◆, From left to right, shrew, rat, human, horse and cow respectively; ▲, marine mammals; ■, porcine. The total sulfatide sulfate per animal (A) and the tissue concentration of the sulfatide sulfate (B) are plotted against body weight.

1. Kidney and Osmoregulatory Organs

(a) Kidney

The kidney of most of the mammals including rat, human, and rabbit contained significant amounts of HSO_3-Chol, and all epithelial cell lines derived from mammalian renal tubules incorporated [^{35}S]sulfate into HSO_3-Chol.[233] The kidney contained the highest concentration of HSO_3-Chol among the visceral organs of adult rats[217,243] and humans.[388] The developmental pattern of HSO_3-Chol in the kidney was quite different from that in the brain and liver. The level of kidney HSO_3-Chol increased steadily from 6.5 nmol/g in 7-day-old rats, reached the adult level of approx. 86 nmol/g in 50-day-olds, and then stayed at the same level.[243] Incorporation of [^{35}S]sulfate into SM4s of mouse kidney peaked between 35 and 42 days of age.[171,233] In contrast, SM4s of mouse kidneys increased sharply after 2 weeks.[93] The major mammalian renal sulfatides are SM4s, whose concentrations are only high in the kidney of mammals next to the nervous tissue.[302] In 1982, the present author[233] began to use symbols SM4s, SB1a, etc. to underline the similarity of the oligosaccharide portion of vertebrate SM4s and the ganglio-series 'sulfatides' enriched in rat kidney to that of GM4 and the ganglio-series gangliosides respectively. The human kidney contains SM3 and sulfatides of globo-series,[400] while the ganglio-, globo- and isoglobo-series sulfatides are found in the rat kidney.[565]

The concentration (nmol/g) of acidic groups of glycolipids in the kidney of mesic mammals is regulated by two principles. The first rule dictates that the amount of glycolipid-bound sulfate (S in μ mol) of mesic terrestrial mammals is correlated with the body mass (M in kg) in the allometric equation $S = aM^b$ where a and b are constants (Fig. 6A). The total glycolipid sulfate (S, μ mol), total lipid-bound sialic acid (N, μ mol), and the total glycolipid anions (the sum of total glycolipid sulfate and ganglioside sialic acid, A, μ mol) per animal were $S = 1.29\ M^{0.737}$ (or $\log S = 0.111 + 0.737 \log M$, correlation coefficient $r^2 = 0.992$), $N = 0.501\ M^{0.846}$ ($r^2 = 0.988$) and $A = 1.85\ M^{0.780}$ ($r^2 = 0.998$) respectively.[398] The total amount of the renal lipid-bound sialic acid above (the exponent 0.846) was directly correlated to the kidney mass because the exponents for the kidney mass and total glomerular volume are about 0.85.[58] In contrast, the exponent for the total glycolipid sulfate was approx. 3/4 being close to those of the biochemical parameters,[59] e.g. the basal metabolic rate (0.76), nitrogen output (0.735); morphological parameters, e.g. the total proximal tubular volume (0.73); as well as the physiological parameters, e.g. the urine production rate (0.75), renal blood flow (RBF, 0.77), and the glomerular filtration rate (GFR, 0.72).

Biometrical handling of metabolic parameters in relation to body mass of animals has been called the allometric law and successfully applied to mammals of various body masses, ranging from a shrew of 2 g to a whale of 30 tons.[58] The above allometric equations can be better represented in μM tissue concentrations of the sulfatide sulfate and the total anionic groups of mammals vs. body mass to cancel the contribution of kidney mass as follows: $S = 0.20\ M^{-0.124}$ ($r^2 = 0.990$)[403] (Fig. 6B). The GalCer sulfotransferase activities in mice (182–550 pmol/hr/mg protein)[93,541] were higher than human activities (42.0 pmol/hr/mg protein),[489] substantiating the higher sulfatide expressions in smaller mammals. These equations were able to correlate the tissue concentration of glycolipids with other biological parameters of the animal for the first time.

The second rule predicts that the proportion of sulfatide sulfates in total anionic groups is progressively lower with increasing body mass. This rule can be derived from the equation of the first rule. The kidney of bovine (650 kg body mass) contained the glycolipid sulfate corresponding to approximately 1/3 of the total anionic groups[398] with the remainder consisting of ganglioside sialic acids. The horse (500 kg), and human (70 kg) contained progressively higher proportions of renal sulfatides corresponding to 2/3, and 3/4 respectively, of total glycolipid acidic groups. Finally, among the smallest mammal examined, Japanese house musk shrew (body mass 0.04 kg), sulfatides occupied about 4/5 (82 mol%) of the renal glycolipid anion, setting the record ever observed in mammalian kidney. This may indicate that sulfate esters function better than sialic acids

as the ion barriers in animals with smaller body mass, which have higher metabolic activities and hence faster urine production per their body mass.[58] In a 'ouabain-resistant' mutant clone of MDCK, the sulfate/sialic acid ratio increased to 61% in comparison to 16% in the wild type MDCK[423] probably compensating the lowered function of the sodium pump.

The concentration of SM4s was higher in the renal medulla of bovine,[265] human,[491] and rabbit,[649] forming a concentration gradient from the cortex to medulla. GalCer and Gal-A_2Gro-sulfotransferase activities were also higher in tubules and medulla.[195] In porcine kidney medulla of male, castrated male and female, the total sulfatide sulfate concentrations (ganglioside sialic acid in parentheses) were 194.0 (51.8), 184.3 (59.4), and 155.9 (49.4) nmol/g respectively, which were 6.1–6.7 times higher than the values for cortex, 32.0 (54.9), 27.5 (59.1), and 24.5 (59.1) nmol/g respectively.[371] The proportion of renal medulla in the whole kidney, represented as the relative medullary thickness (RMT), also increases with decreasing body mass. RMT is an expression of the proportion of the kidney that contains the loop of Henle and collecting ducts, the structure primarily responsible for concentrating urine, to overall kidney size.[59] RMT has a small inverse exponent ($M^{-0.108}$)[34] almost identical to that of the glycolipid sulfate, in whole kidney supporting the significant role of sulfatides in urine concentration.

The sulfatide sulfate occupied 59.7 and 65.3% respectively, of total glycolipid acidic groups in striped dolphin (body mass 140 kg) and sea lion (450 kg).[397] These proportions are higher than those expected from their body masses. In addition, the calculated glycolipid sulfate concentrations in the kidney of terrestrial mesic mammals whose body masses were comparable to those of striped dolphin and sea lion respectively, were 110.3, and 94 nmol/g, whereas the values actually obtained were 133[403] and 261 nmol/g,[397] i.e. more than twice the level calculated for mesic terrestrial mammals. These results indicated that the amount of renal glycolipid sulfate of marine mammals, living solely on metabolic water, was higher than that of mesic mammals. Analogously, the incorporation of [^{35}S]sulfate into SM4s markedly increased in the compensatingly hypertrophied kidney of C_3H/He mice, after the unilateral resection of the kidney.[608]

A terrestrial mesic mammal of the size of porcine (95 kg) should contain 115.5 nmol/g of renal lipid-bound sulfate[371] (Toida, T., Matsumoto, H. and Ishizuka, I. unpublished) as calculated from the allometric equation. The concentrations (in parentheses % in total acidic lipids) actually obtained from male, castrated male and female porcine kidney were 52.1 (48.9%), 47.5 (46.4%) and 40.7 nmol/g (41.3%) respectively. Obviously, these lipid-bound sulfate concentrations were less than the half of the value predicted for mesic mammals, and the molar percentage in acidic lipids lower than that of a horse with the body mass several times larger than the porcine. These results suggested that the low concentration of renal acidic amphiphiles might be closely related to the behavior of pigs (and possibly in beavers) adapted to a water-rich environment where it is not necessary to concentrate urine to preserve water. The porcine has evolved from wild boar (Artiodactyla), and is closely related to hippopotamus of the same subclass suiform, the habitat for both being a place with a plentiful water supply.

The immunofluorescence observation using a polyclonal antibody against SM4s suggested that SM4s in the rat kidney distributed predominantly at luminal (apical) membrane of the thick ascending limb in contrast to Na^+,K^+-ATPase that was known to locate at basolateral membrane.[649] MDCK cells, assumed to originate from collecting tubules based on hormone sensitivities,[235] contained high concentrations of SM4s in contradiction to the negative staining of rabbit collecting tubules for SM4s.[649] Histological studies using a specific monoclonal antibody,[56] and an L-selectin analogue[581] supported the tubular localization of sulfatides. Injection of a monoclonal antibody against sulfatides, Sulph I, into mice and rats showed homing to kidney tubules.[57] Kidney sections of rats, mice, pigs and monkeys were also intensely stained at the wall of the juxtaglomerular arterioles, and at the macula densa area, with Sulph I.[56] Rat or rabbit sera of Masugi's or Heymann's nephritis produced by immunization with proximal tubular

homogenate contained two populations of antibodies, specific to SB2 and SM2a respectively.[246]

In mesic species, the exponents for relative medullary thickness (RMT) ($M^{-0.108}$),[34, 58, 59] the maximum urine concentrating ability ($M^{-0.097}$)[34] and the metabolic rate for kidney cortex slices ($M^{-0.13}$)[79] are close to the allometric slope, $M^{-0.124}$, of the renal sulfatide sulfate. Thus the kidney of smaller animals filters at a higher rate per gram, in other words, it produces urine at a higher rate per gram.[58] Electron microscopy has shown that smaller animals has more extensive basolateral infoldings.[5] Basolateral membrane area per unit medullary thick ascending limb (mTAL) cellular volume ($M^{-0.075}$), as well as the volume of mitochondria as a percent of mTAL cellular volume ($M^{-0.056}$) increased with decreasing body mass. Thus, not only do mitochondria occupy more volume of mTAL cells, but those mitochondria are also more densely packed with cristae of smaller mammals.[5] Inner mitochondrial membrane area per unit volume of mTAL cell cytoplasm scaled as $M^{-0.092}$, in reasonable agreement with the concentration of sulfatide sulfate (−0.124). The exponents for the filtration performed per gram of kidney, and the potassium uptake rate[79] were similar (1.2 $M^{-0.13}$, and 0.73 $M^{-0.13}$), confirming the stoichiometry between the glycolipid sulfate and the activities of Na^+,K^+-ATPase.[270, 514]

(b) Cultured renal cells

Well-differentiated epithelial cell lines derived from various segments of the renal tubule, MDCK (canine), JTC-12 (cynomolgous monkey), LLC-PK_1 (porcine), and MDBK (bovine), contained sulfatides.[235, 423, 555] SM3, and SM2a were often observed at high levels in the cultured cell lines, although not detectable or detected at quite low levels in the original tissues.[233] The profile of glycosphingolipids of MDCK and Verots cells in culture with anisosmotic media indicated that a hyposmotic medium reduced the concentrations of SM4s, SM3,[424] and SB1a.[425] On the contrary, the concentrations of sulfatides increased by maintaining the culture in hyperosmotic media, while the contents of most of the neutral glycolipids decreased. The hyperosmotic medium supplemented with nonelectrolytes, mannitol, sucrose or urea, also elevated the concentration of sulfatides.

The incorporation of radioactive sulfate into sulfatides[424] and the activity of GalCer sulfotransferase[231] were elevated in cells of renal tubular origin, JTC-12, LLC-PK_1, MDBK,[424] and Verots,[425] adapted to high NaCl or mannitol. The *in vitro* stimulation of sulfotransferase at NaCl concentrations much higher than the intracellular environment[555] may serve as an explanation for the stimulation of sulfatide synthesis in hyperosmolar media.[231] It has been established that the amount and turnover rate of sulfatides increases in adaptation to hyperosmolality by the intrinsic cellular mechanisms independent of the integral regulatory mechanisms including hormones, and the elevation of the synthesis is primarily responsible to the local barrier requirements of individual cell.[426] Two clones (osmR-A and osmR-B), resistant to hyperosmotic media of 700 and 800 mosM/l respectively, were selected from MDCK cells. Even when cultured in isosmotic medium (300 mosM/l), the concentration of SM4s and SM3 in these hyperosmosis-resistant clones was 3.4–5.9 times higher than that in the wild-type MDCK. The rate of incorporation of [^{35}S]sulfate into sulfatides of osmR-A and osmR-B was also 1.9–6.7 times higher than that of MDCK.[426]

(c) Other osmoregulatory organs

Both HSO_3-Chol and SM4s were highly concentrated in the tracheal epithelia of rabbit.[464] HSO_3-Chol,[243] and SM4s[645] were also contained in the stratum corneum of the skin, which functions primarily as a barrier against transepidermal water loss[338, 661] and protects internal organs from dessication. HSO_3-Chol, GlcCer and *N*-(*O*-linoleoyl)-ω-hydroxy fatty acyl GlcSph constituted comparable concentrations in the skin and epidermis of mammals and increased concurrently during the development.[251] Rabbit tracheal epithelial cells in primary culture undergo the terminal differentiation at confluence, with

concomitant accumulation of HSO_3-Chol by the cells, to yield cornified cells much in analogy to epidermal keratinocytes. Squamous differentiated tracheal cells also exhibited 20- to 30-fold higher levels of cholesterol sulfotransferase activity than those in undifferentiated cells did.[464] Cholesterol sulfotransferase was concentrated in the basal and spinous layers of the skin, whereas arylsulfatase C, which hydrolyzes HSO_3-Chol, was rich in the stratum corneum and the granular layer.[384] Guinea pig oral and nasal mucous membranes, which function as the first protective barrier against inhaled air, contained even higher concentrations of HSO_3-Chol than those in tracheal mucosa with higher cholesterol sulfotransferase and lower cholesterol sulfatase activities.[199]

Aqueous humor is actively secreted from the basal surface of the epithelial cells of the ocular ciliary bodies, which participate in ion transport and osmoregulation. Electron microscopic autoradiography of the ciliary body epithelium (earlier these authors used 'ciliary processes') of rat eyes showed the synthesis of SM4s.[32] In addition, an anti-SM4s monoclonal antibody stained the scattered spot-like structures in the choroid layer of murine and human eye,[56] especially pericytes, which play a role in the development of diabetic retinopathy.[55]

2. *Nervous System*

HSO_3-Chol,[243] SM4s,[302] SM4g,[238,395] SMUnLc$_4$Cer, and SMUnLc$_6$Cer[249] are the major sulfoamphiphiles of the mammalian nervous system. SM4s in the brain increased throughout the major portion of the life of man and some animals parallel to the total polar lipid of various regions.[480]

(a) Central nervous system

The age-dependent change of the HSO_3-Chol concentration in rat brain approximately paralleled the peak of myelination, although the subcellular distribution revealed that HSO_3-Chol was enriched in the nerve ending fraction (synaptic junctions).[243] However, intracranial[111] or intraperitoneal[228] administration of $[^{35}S]O_4$ did not label HSO_3-Chol of rat brain.[111] Fractionation of rat brain cells indicated that SM4s was concentrated in the myelin and oligodendrocytes, the cell producing myelin[461,618] but not in neurons and astroglia.[3,122] It has been histologically confirmed by using monoclonal antibodies, that SM4s is distributed in myelin[543] and oligodendrocytes[122,309] in rat brain. Both GalCer and SM4s were not expressed on astrocytes,[122,309] whereas most of the astrocytoma tissues contained both GalCer and SM4s.[245] Cell lines derived from astrocytoma and neuroblastoma contained only negligible amounts of SM4s[85] and did not incorporate $[^{35}S]$ into SM4s. On the other hand, the frequency of staining for SM4s with the monoclonal antibody O4 indicated that low grade and anaplastic astrocytomas, classified on histological grounds as astrocytic, are often stained with antibodies that recognize oligodendrocytes and their progenitors.[346] SM4s constitutes approx. 1/5 by weight of the glycolipids and 6 mol% of total lipids in the myelin membrane of the adult mammalian central nervous system and has a very slow turnover.[54,248] The concentrations of SM4s were high in the order of brain stem > diencephalone > cerebellum > cerebrum, depending on the amount of myelin-rich nerve fibers.[431] GalCer and SM4s may be the indispensable components of myelin because mice lacking in GalCer and SM4s exhibit severe generalized tremoring after 16 days of age due to reduced action potentials.[75]

The specific radioactivity of the lipid $[^{35}S]$ in myelin-enriched fraction increased to about 15-fold[226] of the activity in the homogenate of whole brain 24 hr after the intraperitoneal injection of inorganic $[^{35}S]$sulfate to 17-day old rats. The relative specific activity of sulfatides in myelin fraction was about 50, whereas those of other fractions, i.e. nuclei, synaptosomes, mitochondria and cytosol, were less than 1.0 indicating that SM4g was most enriched in the myelin fraction. Although SM4g has been thought to be a minor component of adult mammalian brain, it occupies approx. 42 mol% of the total cerebellar sulfatides in 14-day-old mouse[52] and 16% of total sulfoglycolipids

of rat brain at the peak of myelination (18–21 days)[228,238,395,452,525] but only 8.4% in adult (3–4 months) rats.[452] In the culture of embryonal mouse brain cells, the incorporation of [^{35}S]sulfate into SM4g was 17–21% of total sulfoglycolipids.[36] The Jimpy,[452] and *qk/qk* mice,[306] deficient in myelin formation, showed reduced brain sulfotransferase activities, although the level of SM4g in testes was normal indicating that this mutation does not affect sulfatide levels in other organs.[306] The SM4s concentration of cerebrospinal fluid (CSF)[84] in patients with vascular dementia (VAD)[124] and HIV[142] was significantly higher than that in controls and Alzheimer disease, reflecting demyelination and damage of blood–brain barrier respectively.[122] The values for gangliosides and SM4s of CSF in children and adults,[84] as well as in animals[122] increased during development and aging.

The age for the most active synthesis of SM4s in mouse brain was 20 days[54,233] coinciding with the peak of myelination, whereas SM4g was synthesized with a peak around 14 days.[52] The peak of these parameters in rat brain occurred a few days of age later[228] and closely associated with the age of the most active myelination.[228,452,542] After the age of active myelination, SM4s in rat[227] and shrew[624] continued to increase until 310 and 70 days respectively, in parallel with other typical myelin components including GalCer, sphingomyelin and phosphatidylinositol bisphosphate. The levels of the major galactolipids of rat spinal cord, GalCer and SM4s, increased linearly during the first two months after birth. At three months of age, constant levels were reached which were approx. 4-fold (GalCer) and 2.5-fold (SM4s) higher than in cerebral tissue of corresponding age.[88]

SM4g and its synthetic activity were barely detectable in the brain of SD rat before 10 days of age,[452] and even in 14- to 16-day Wistar rats.[228] Accumulation of SM4g, predominantly A_2Gro form, began with the onset of myelination (19 days), whereas after 22 days EAGro form was the major molecular species.[228] SM4g then continued to increase in close parallel with SM4s until 31 days of age, reached a plateau at 48 days, then decreased dramatically after 68 days of age[227,228] while SM4s continues to increase. SM4s in myelin is one of the most metabolically stable components of mammals whereas SM4g turns over slightly more rapidly.[52,224,248] The longer term experiment *in vivo* showed that A_2Gro form of SM4g in rat brain diminished more rapidly than the EAGro form.[227,452] Only about 1/10 (21.2 nmol/brain) of the level of SM4g at 48 days of age (total of both forms, 213 nmol/brain) remained at the age of 175 days and was not detected radiochemically and chemically at 310 days.[227] SM4g (EAGro form) accumulated in rat spinal cord[88] in a similar fashion as Gal-A_2Gro but did not drop dramatically after 70 days as that in cerebrum. In primary cultures initiated from 19–21-day-old dissociated fetal SD rat brain,[521] GalCer, SM4s, and Galβ-A_2Gro were synthesized and accumulated by 8 days in culture. Thereafter the synthetic rates and levels of these glycolipids increased rapidly, reaching maximal values between 22–29 days in culture. The percentage of [^{35}S]sulfate incorporated into SM4g declined from 15% at day 2 *in vitro* to 5% at day 12.[304]

Sulfated glucuronyl neolacto-series sphingoglycolipids, SMUnLc$_4$Cer and SMUnLc$_6$Cer, were identified as the antigen of neuropathy IgM paraprotein.[221] SMUnLc$_4$Cer and SMUnLc$_6$Cer were localized predominantly in the postmigratory neuronal cells[381] as well as in BMEC (cultured bovine brain microvascular endothelial cells).[261] They may probably be related to external granulocytes of developing rat and the molecular layer containing dendrites of Purkinje cells in the adult rat cerebrum and cerebellum respectively.[69,71] Significant reduction in the content of the sulfoglucuronylneolacto series glycolipids[65] and *N*-acetylglucosaminyltransferase activity[71] was observed in the cerebellum of the Purkinje cell abnormality mutant mice including Purkinje cell degeneration (*pcd/pcd*), lurcher (*Lc/+*), and staggerer (sg/sg). SMUnLc$_4$Cer and SMUnLc$_6$Cer were present during the embryonic stage of development of the forebrain[381] and in nerve growth cone membranes from fetal rat forebrain or brainstem[214] but disappeared soon after birth.

(b) Peripheral nervous system

Human peripheral nerve myelin from both sensory and motor nerves contained 1.1–2.5% of SM4s in the total lipid extract[434] or 50 nmol/g.[122,552] Mouse monoclonal antibodies Sulf I[55,56] and 224–58[150] interacted specifically with SM4s and SM4g in the membrane of non-motor peripheral nerves including the vagal nerve and Schwann

Table 3. Distribution of sulfoamphiphiles in the biosphere

Prokaryotes and plants
HSO_3-PtdGro ($E_{20}E_{20}$ analogue), *Haloarcula marismortui*, 17 mol% of polar lipids.[100] *Halobacterium salinarum* (*cutirubrum*), 4 mol% of polar lipids.[326] *Hb. sodomense*, 3% of the total lipid.[256,597] *Hb. trapanicum*, 15 weight % of polar lipids[256,598]
HSO_3-6Glcα-1$E_{20}E_{20}$Gro, chemical synthesis[257]
HSO_3-6Manpα-2Glcpα-1$E_{20}E_{20}$Gro (S-DGD-1), *Haloferax mediterranei* (strain R-4), 2.8 μ mol/g (25 mol% of polar lipids).[326] *Hb. saccharovorum*, 13% of the total lipid.[256,326] *Halococcus saccharolyticus* strain, 19.5 mol% of polar lipids.[383] *Hb. salinarum*, (+).[368] Chemical synthesis[257]
HSO_3-2Manα-4Glcα-1$E_{20}E_{20}$Gro (S-DGD-3), *Hb. sodomense*, (+)[256,597]
HSO_3-2Manα-2Glcα-1$E_{20}E_{20}$Gro (S-DGD-5), *Hb. trapanicum*, 29% by weight of polar lipids[598]
HSO_3-3Galpβ-6Manpα-2Glcpα-1$E_{20}E_{20}$ Gro (S-TGD-1), *Hb. salinarum* (*cutirubrum* or *halobium*), 24% of polar lipids;[256,271,275] 21%;[326] FAB, ^{1}H-NMR, (+).[102] *Hb. saccharovorum*, (+)[256,325]
HSO_3-3Galβp-6(Galfα-3)Manpα-2Glcpα -1$E_{20}E_{20}$Gro (S-TeGD), *Hb. salinarum*, (+).[527] *Hb. saccharovorum*, (+)[256,325]
$(HSO_3)_2$-2,6Manα-2Glcα-1$E_{20}E_{20}$Gro and $E_{25}E_{20}$Gro (S_2-DGD-1), halophilic bacterium *Natrialba asiatica* (former strain 172), (+).[369] Strain B1T, (+)[256]
2,3,6,6′-Tetraacyl-trehalose-2′-sulfate, Mycobacteria, (+)[149,159]
SQ-A_2Gro (17:0 branch), *Sulfolobus acidocaldarius* (glycolipid K), 9% of the total lipid or 43% of acidic lipids.[332] SQ-A_2Gro [16:0, 18:0cyc, 18:1(ω7)], freshwater bacterium *Caulobacter bacteroides*, 9% of the total lipid.[665] SQ-A_2Gro, anaerobic photosynthetic bacterium *Rhodopseudomonas viridis*, (-).[666] SQ-A_2Gro (16:0 and 18:1), photosynthetic (marine green) alga *Enteromorpha flexuosa*, 67 nmol/g (200 μg/g dry cell).[518] SQ-A_2Gro (18:3), green leaves of *Vicia faba*, 290 nmol/g.[284] The thylakoid membrane of chloroplasts, 70 μ mol/g.[238] *Rhodobacter spheroides*, cultured in 1.0 mM, Pi, 2.2 mol% of the total lipid; in 0.1 mM Pi, 16.6 mol%[31,155]
6-Sulfoquinovosyl-3′,2′-*O*-acyl-1′-*O*-thioacyl-*sn*-Gro (thionsulfonolipid), a picoplankton cyanobacterium (*Synechococcus* sp.), 670 nmol/g (0.2% of dry cells)[281]
Taurine-6GlcUα-3-*sn*-A_2Gro, *Hyphomonas jannaschiana* (a seawater bacterium), 34% of the total lipid or 18 μ mol/g (52 mg/g dry cell)[29]
$(HSO_3)_2$-3,4-(3-*O*-methyl-1-oxobutyl)-2Glcpβ-[15-hydroxy-2-oxy]-19-norkaur-16-en-18oic acid (atractyloside), *Atractylis gummifera*, (+)[590]
$(HSO_3)_2$-1,12-docosanediol, a phytoflagellate *Ochromonas danica*, (+)[159]
HSO_3-1Cer (d18:1/24:0), chemical synthesis[430]
HSO_2-1Cer (d18:1/16:1-Δ^3-*trans*), a nonphotosynthetic diatom *Nitzschia alba*, (+)[12]
HSO_3-3(24-methylene)Chol, *Nitzschia alba*, (+)[12]
Invertebrates, fishes, amphibia, reptiles and birds
HSO_3-Chol, sea star *Asterias rubens*, 560 nmol/g (1.3 mg/g dry tissue).[39] Sea urchin *Anthocidaris crassispina*, 2.2 μ mol/g (1.04 mg/g, 15% of polar lipids).[647] Chemical synthesis[607,647]
SQ-A_2Gro, sea urchin *Pseudocentrotus depressus*, eggs, 300 nmol/g; spermatozoa, 700 nmol/g.[239] SQ-A_2Gro (16:0), sea urchin *Anthocidaris crassispina*, shells, 30 nmol/g[287]
SM4s, orange star fish, radial nerve, trace; SM4s (2-hydroxy acid), amphioxus, ventral nerve, 0.8 nmol/g[441]
HSO_3-8NeuGcα2-6GlcCer (t18:0/15:0 and t18:0/22h:0), sea urchin gonads, (+).[297] Sea urchin eggs (t18:0/24h:0), 15 nmol/g (94 μg/g dry eggs)[315]
HSO_3-8NeuGcα2-6Glc-8NeuGcα2-6GlcCer, sea urchin eggs, (+)[457]
HSO_3-8NeuAcα2(-8NeuAcα2)$_n$-6GlcCer (t18:0/20:1,21:1,22:1) (n = 0,1,2, and 3), male sea urchin gonads, < 38 nmol/g[218]
SM4s (d18:1/24:1 and d18:1/24h:1), a coelacanth *Latimeria chalumnae*, brain, 3 μ mol/g[579]
SM4s (d18:1, t18:0/22h:0, 24 h:1), spiny dogfish, salt (rectal) gland, 640 nmol/g (2.8 mg/g dry tissue), 470 nmol/g (determined by hexose analysis on the pure lipid fraction, 2.1 mg/g dry tissue).[269] SM4s (d18:1/24:1 and d18:1/24h:1), a ray *Torpedo marmorata*, electric organ, 1180 nmol/g (5.9 μ mol/g dry tissue).[174] SM4s, a shark *C. longimanus*, brain, 3.1 μ mol/g (2.8 mg/g tissue).[312] Brown shark, brain, 3.6 μ mol/g.[441] Skate fish testis, TLC (+)[342]
SM4s, eel (*Anguilla anguilla*), gills, [^{35}S]labeling/TLC, adapted to fresh water, 45 nmol/g; seawater, 59 nmol/g[654]
SM4s, teleost fishes, brain, 1.0–2.1 μ mol/g (0.9–1.9 mg/g).[312] SM4s (fatty acids are exclusively nonhydroxy), Alaskan pollack, brain, 1350 nmol/g (12 mol% of total glycolipids); spinal cord, 2660 nmol/g (13 mol%).[576,577] SM4s (nonhydroxy fatty acids, 90%), common carp, brain, 370 nmol/g (20 mol%).[577] SM4s (nonhydroxy fatty acids predominate), killifish, brain, 1.6 μ mol/g; spinal cord, 1.4 μ mol/g.[441] SM4s (d20:1/24:1), puffer (*Fugu rubripes rubripes*), testis, 17 nmol/g (15 μg/g).[605] Medaka (teleost), skin and alimentary tract, TLC-OL, SM4s, (+).[113] Cod intestine, TLC (chemical staining), SM4s (–)[47]

—continued

Table 3—*continued*

SM4g, puffer, testis, (–).[605] Alaskan pollack, brain, 1690 nmol/g (15 mol% of total glycolipids); spinal cord, 2580 nmol/g (13 mol%); common carp, brain, 0 nmol/g[577]

SM3, testes of rainbow trout and salmon, TLC, (+).[342] SM3 (d18:1/16:0), salmon milt, 426 nmol/g (2 mg/g dry weight)[347]

SM4s (d18:1/24:1), bullfrog tadpole, metamorphosis XI–XVI, 250 nmol/g; bullfrog brain, 790 nmol/g; SM4s (d18:1/24:1), sciatic nerve, 1350 nmol/g.[485] SM4s (d18:0/24h:1), adult frog brain, 800 nmol/g.[578] SM4s (d18:0/24:1), axolotl brain, neotenous adult form, 560 nmol/g; metamorphosed adult form, 710 nmol/g.[578] SM4s, Chilean frog (*Calyptocephalella caudiverbera*) skin, tadpole of 31 day, 210 nmol/g (2.0 mg/mg protein); 44-day tadpole, 640 nmol/g (6.2 mg/mg protein)[651]

SM4s, toad, convoluted oviduct, Azure A assay of the total organic phase lipid, 150 nmol/g (134 μg/g) in March, 260 nmol/g (232 μg/g) in May; uterine oviduct, 540 nmol/g (487 μg/g) in March, 750 nmol/g in May (671 μg/g).[615] Xenopus, ovaries, > 85% of the total acidic glycolipids[316]

SM4s (d20:1/22-24h:0), duck, salt gland, adapted to fresh water, 230 nmol/g; adapted to saline, 810 nmol/g.[268] SM4s (d20:1/22-24h:0), eider duck, salt gland, 1.64 μ mol/g; herring gull, salt gland, 1.13 μ mol/g.[270] Mature fowl testis, (+); immature duck testis, (±); mature duck testis, (+ +).[342] SM4s, adult chicken (> 70 days after hatching), retina, 24 nmol/retina or 295 nmol/g; brain, 2350 nmol/g[95]

SM4g, adult chicken, 1.8 nmol/retina[95]

Mammalian

Kidney

HSO_3-Chol, rat (SD), hydrolysis and GC of the fraction purified on silicic acid columns, 107–132 nmol/g (249–307 μg/g dry tissue);[243] Wistar rat, $FeCl_3$ assay of the fraction purified on DEAE–Sephadex and silicic acid columns, 89 nmol/g.[217] Rabbit, 275 nmol/g (2.75 nmol/mg protein)[81]

Sheep, (+).[299] Human, 430 nmol/g (200 μg/g); metachromatic leukodystrophy patient, 3.5 y, approx. 430 nmol/g.[388] Human urine, 0.4 nmol/ml.[41] Monkey renal cell line JTC-12 and canine kidney cell line MDCK,[35] S-label, (+)[228,233,423]

SM4s (d18:1/22h:0, 24 h:0), GC with mannitol as an internal standard, house musk shrew (*Suncus murinus*), 299 nmol/g (82 mol% of the total acidic glycolipid.[398] SM4s, mouse (C57BL/6J-*cpk*, 3 weeks), densitometry after charring the TLC plate with copper sulfate/phosphoric acid, 457 nmol/g; cortex, 373 nmol/g; medulla, 935 nmol/g; cystic kidney, 34.4 nmol/g.[93] Prosaposin-deficient mouse, 5-fold increase[130]

SM4s (d18:1/22-24h:0), rat (Wistar), GC using mannitol as an internal standard, 182 nmol/g.[559] SD rat (30–120 day), HPLC of benzoylated desulfated SM4s (d18:1/hydroxy fatty acids), 140–260 nmol/g (0.7–1.3 nmol/mg dry weight); SM4s (d18:1/nonhydroxy fatty acids), 60–160 nmol/g (0.3–0.8 nmol/mg dry tissue).[515] SM4s, 60-day-old SD rat (body weight 200 g), TLC densitometry of the purified SM4s after orcinol stain, 201 nmol/g.[217] Lewis rat, TLC-OL using the monoclonal antibody and densitometry, 278 nmol/g; rat and other mammals, distal tubules, (+).[56] Rat kidney Golgi complex fraction, 13 μ mol/g (117 μg/mg protein); plasma membrane fraction, 6.1 μ mol/g (55 μg/mg protein)[650]

SM4s, rabbit glomeruli, preparative TLC, 16 nmol/g (8 nmol/100 mg dry weight); cortex, 26 nmol/g (13 nmol); cortical tubule, 54 nmol/g (27 nmol); medulla, 208 nmol/g (104 nmol);[649] rabbit (6 month, New Zealand White) kidney, 96 nmol/g (0.96 nmol/mg protein)[81]

SM4s, human (body weight 69 kg), DEAE-cellulose column, 81–112 nmol/g;[366] 120 nmol/g (0.54 mg/g dry tissue).[266] SM4s (d18:1/22:0), human, cortex, 59 nmol/g; SM4s (d18:1/24h:1), medulla, 169 nmol/g.[491] SM4s, human fetal (20 weeks), TLC densitometry, normal, 44 nmol/g (0.22 nmol/mg dry tissue); Krabbe disease, 78 nmol/g (0.39 nmol); Sandhoff disease, 8 nmol/g (0.04 nmol); metachromatic leukodystrophy, 52 nmol/g (0.26 nmol); prosaposin deficiency, 196 nmol/g (0.98 nmol).[46] SM4s, elevated in a patient of atypical Farber disease.[131] Normal human urine, perbezoylation and desulfation, 0.16 nmol/mg creatinine (67% in the sediment), urine of metachromatic leukodystrophy patients, 7.6 nmol/mg creatinine[415]

SM4s (d18:1/23h:0,24 h:0), sheep, preparative TLC and gas chromatography, 22 nmol/g.[299] SM4s (d18:1/23h:0, 24 h:0), horse (body weight 485 kg), 99.8 nmol/g;[599] SM4s (d18:1, t18:0/23h:0, 24 h:0), (+).[181,570] SM4s (d18:1/24h:0), bovine (body weight 650 kg), 86 nmol/g (0.4 mg/g dry tissue); cortex, 22 nmol/g (0.1 mg/g); transition zone, 65 nmol/g (0.3 mg/g); medulla, 194 nmol/g (0.9 mg/g); large papilla, 86 nmol/g (0.4 mg/g)[265]

SM4s, porcine (body weight 95–105 kg), male, renal cortex, 31.3 nmol/g, medulla, 193 nmol/g; castrated male, cortex, 26.0 nmol/g, medulla, 183 nmol/g; female, cortex, 23.6 nmol/g, medulla, 155 nmol/g[371] (Toida T., Matsumoto H., and Ishizuka, I., unpublished results)

SM4s (d18:1/22h:0), dolphin *Stenella coeruleoalba* (body weight 140 kg), 124 nmol/g.[401] SM4s (d18:1/22h:0), a sea lion *Eumetopias jubata* (suborder *Pinnipedia*, body weight 450 kg), 252 nmol/g[397]

SM4s, human renal cell carcinoma (Grawitz) tissue (adenocarcinoma), 1.0–72.0-fold the level in the uninvolved tissue.[490] Renal cell carcinoma, 1.7-fold of the uninvolved tissue; Wilms' tumor, (–).[489] SMKT-R3 human renal carcinoma cells, cytofluorometry, (+)[293]

SM4s, JTC-12 cell line, 40 nmol/g (20 nmol/100 mg dry weight).[225,234] SM4s (d18:1/16:0), MDCK, 70 nmol/g (0.7 nmol/mg protein); MDCK, a 'ouabain-resistant' mutant, 190 nmol/g.[423] SM4s + ^{35}S-label, MDCK strain I, (+); MDCK strain II, (+ + +)[421]. MDCK strain I, (+); MDCK strain II, (–).[176] MDCK strain II, [^{35}S]sulfate and [^{3}H]ceramide labeling, (+).[657] MDCK cultured for 3–7 days in a hyposmotic medium (100 mosM/l), 46 nmol/g; cultured in a hypertonic medium (500 mosM/l), 93 nmol/g; in a medium made to 500 mosM/l by additions of mannitol, 120 nmol/g.[424] MDCK, 23 nmol/10^8 cells; fraction insoluble in Triton X-100, 13 nmol/10^8 cells.[49] SM4s (d18:1/18:0), SGE1 rat renal glomerular epithelial cells, 8.0 ± 4.3 nmol/mg DNA; monolayer with domes 9.6 ± 1.2 nmol.[595] EUE (human embryonic epithelium) line, Azure A assay of organic phase lipids, in the isotonic medium, 2.9 μ mol/g (28.0 μg/mg protein); 12.6 μg/g (120 mg/mg protein).[42] Human renal cancer cell line SMKT-R3, Azure A staining, TLC-OL with monoclonal antibody Sulf I, [^{35}S]-labeling, (+)[293]

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Table 3—*continued*

SM4s-Glc (t18:0/24:0), Wistar rat kidney, TLC densitometry (orcinol), 5.5 nmol/g.[217] SM4s-Glc, MDCK, DEAE-Sephadex column, (+)[426]
SM4g, SD rat kidney, spleen, intestine, and liver, [^{35}S]-labeling, (–).[354] Wistar rat kidney, (–)[559]
SM3 (d18:1/18:0), house musk shrew, 0.5 nmol/g.[398] SM3, prosaposin-deficient mouse, mildly elevated[130]
SM3 (t18:0/22–24:0), rat, 1.7 nmol/g;[559] 1.6 nmol/g.[217] Human kidney, 30 nmol/g.[366,535] SM3 (d18:1/24:0), human, cortex, 33 nmol/g; medulla, 11 nmol/g.[491] Human renal cell carcinoma (Grawitz) tissue (adenocarcinoma), 1.3–39.4-fold the level in the uninvolved tissue;[490] 86.5-fold the level in the uninvolved tissue.[489] Human Wilms' tumor, (–).[489] SM3 (d18:1/22:0), dolphin *Stenella coeruleoalba*, 8.7 nmol/g.[401] SM3 (d18:1 and t18:0/22:0), a sea lion *Eumetopias jubata*, 9 nmol/g.[397] Sheep, 3.3 nmol/g[299]
SM3, JTC-12, 53 nmol/g (0.53 nmol/mg protein).[225,234] SM3 (d18:1/16:0), MDCK, 20 nmol/g; MDCK, a 'ouabain-resistant' mutant, 40 nmol/g.[423] MDCK cultured for 3–7 days in a hyposmotic medium (100 mosM/l), 18 nmol/g; cultured in a hypertonic medium (500 mosM/l), 54 nmol/g; in a medium made to 500 mosM/l by additions of mannitol, 50 nmol/g.[424] MDCK strain II, [^{35}S]sulfate and [^{3}H]ceramide labeling, (+).[657] MDBK,^{35} S-label, (+) as the major sulfatide.[233,234,424] Human renal cancer cell line SMKT-R3, Azure A staining, TLC-OL with monoclonal antibody Sulf I, [^{35}S]-labeling, (+);[293] Verots, [^{35}S]-labeling, (+)[425]
SM2a (t18:0/24:0), Wistar rat, 24 nmol/g.[559] SM2a, SD rat, (+).[246] Human, (–).[399] SM2a, JTC-12,^{35} S-label, (+);[230,424,555] porcine kidney cell line LLC-PK$_1$,^{35} S-label, (+);[424] Verots,^{35} S-label, (+);[425] human renal cancer cell line SMKT-R3, Azure A staining, [^{35}S]-labeling, (+)[293]
SM2b (t18:0/24:0, 22:0), SD rat, 0.05 nmol/g.[565] Human renal cancer cell line SMKT-R3, [^{35}S]PAPS-labeling, (+) (*in vitro* formation)[292]
SB2 (t18:0/24:0), Wistar rat kidney, 11 nmol/g.[560] SD rat, approx. 7.5 nmol/g.[246] Human renal cancer cell line SMKT-R3, [^{35}S]PAPS-labeling, (+) (*in vitro* formation)[292]
SB1a (t18:0/24:0), Wistar rat, 6 nmol/g.[561] SB1a, porcine, (+)[371]
SMGb$_4$Cer (d18:1/24:1, t18:0/24:1), human, 0.03 nmol/g.[400] SD rat, 0.07 nmol/g[564]
SMiGb$_4$Cer (t18:0/24:0), SD rat, 0.27 nmol/g[564]
SMGb$_5$Cer (d18:1/24:0), human, 0.19 nmol/g[399]
SMiGb$_5$Cer (t18:0/24:0 and 22:0), SD rat, 0.11 nmol/g[568]
SMGM1a (II^3-NeuGc,IV^3-HSO$_3$-Gg$_4$Cer) (t18:0/24:0), SD rat, 120 pmol/g[567]

Epidermis, keratinocytes, and eyes
HSO$_3$-Chol, guinea pig, dorsal epidermis, 790 nmol/g; dorsal dermis, 120 nmol/g.[604] Murine skin, 15-day-fetus, 39 nmol/g (0.09 μg/mg dry weight); epidermis, 17-day-fetus, 211 nmol/g (0.49 μg/mg dry weight).[251] Human cohesive stratum corneum, upper arm, 1.2 ± 0.5%; palm, 1.3 ± 1.2% of the total lipid.[512] X-linked ichthyosis patient, stratum corneum, elevated.[630] Normal human, scale, 2.3% of the total lipid; X-linked ichthyosis 12.2%.[630] Bovine hoof, (+).[607] Horse hoof, 19.6% of horse hoof lipids, 10% of cow hoof lipids[626]
SM4s, guinea pig, dorsal epidermis, (–); dorsal dermis, 6 nmol/g[604]. Human keratinocytes, Sulf-1 imunohistochemistry, (±).[154] Immunohistochemistry, (+) both acinar and ductal cells of the normal human mammary glands.[22] H3630 breast carcinoma cell line;[17] human breast ductal carcinoma cells, [^{35}S]incorporation, (+)[583]
SM4s, rat, ciliary body epithelium, basal membrane,^{35} S incorporation, (+).[32] Choreoidea of the eye, human, immunohistochemistry and TLC-OL, 30 nmol/g; Lewis rat and SD rat, 4.8 nmol/g, (+) pericytes[55]
SM3, human breast ductal carcinoma cells, [^{35}S]incorporation, (+)[583]

Lung
HSO$_3$-Chol, rat (43-day-old), lung, 21.3–24.5 nmol/g (49.5—57.0 μg/g dry tissue).[243] Rat lung, [^{35}S]-labeling, (+).[354] Guinea pig, nasal mucosa, 136 nmol/g (0.68 μ mol/g dry weight); oral mucosa, 194 nmol/g (0.97 μ mol); tracheal mucosa, 26 nmol/g (0.13 μ mol).[199] Rabbit, tracheal epithelial cells, (+)[464]
SM4s, rat lung, undifferentiated small cell carcinoma, 2 nmol/g (11 μg/g dry tissue); adenocarcinoma, 8 nmol/g (36 μg/g dry tissue); human lung, 0.7 nmol/g (3 μg/g dry tissue); embryonal lung, 4 nmol/g (20 μg/g dry tissue); SM4s (d18:1/24:0) squamous cell carcinoma, 0.9 nmol/g (4 μg/g dry tissue).[138,645] Immunohistochemistry, (+), human lung adenocarcinoma, 17 in 43 cases; large cell carcinoma, 5 in 25 cases.[378] Small cell carcinoma, Azure A staining, (+)[144]
SM4s, porcine respiratory ciliated cells, (+)[652]

Central nervous system
HSO$_3$-Chol, brain, human, 4-day-old, (+);[388] SD rat, 7.0–9.0 nmol/g (16–21 μg/g dry tissue)[243]
SM4s, house musk shrew (*Suncus murinus*), 70 days, Azure A assay of the acidic glycolipids, cerebrum, 3.0 μ mol/g; cerebellum, 4.2 μ mol/g; bulbus olfactorius, 3.5 μ mol/g[624]
SM4s, brain, mouse (16 days), 1017 nmol/g; *msd*, a myelin-deficient mutant, 365 nmol/g; reduced in other myelin-deficient mutants, *jp* and *qk*.[375] Cerebellum, normal mouse, 1.9 μ mol/g; *Lc*/+ mutant mouse (Purkinje cell degeneration), 3.9 μ mol/g; *wv*/*wv* mouse (Purkinje cell defect), 2.8 μ mol/g; *sg*/*sg* mouse, 3.2 μ mol/g; control of *rl*/*rl*, 2.9 μ mol/g; *rl*/*rl* mutant, 3.5 μ mol/g; *qk*/*qk* myelin-deficient mutant, 0.34 μ mol/g.[66] Mice lacking in UDP-Gal: Cer galactosyltransferase, (–).[75] Mouse (32 days), cerebellum, 6 μ mol/g[54]

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Table 3—*continued*

SM4s, SD rat (22 day), brain, 2.0 μ mol/g; 180 day, 4. 8 μ mol/g;[24] 120 day brain, SM4s (hydroxy fatty acid), 1.9 μ mol/g; SM4s (nonhydroxy fatty acid), 1.9 μ mol/g.[515] SM4s (d18:1/18:0, 24 h:0), SD rat, 15–20 days, silicic acid column and preparative TLC, myelin, 6.9 μ mol/g (4.4% of dry weight); astroglia, 0.2 μ mol/g (0.2%); neurons, 0.05 μ mol/g (0.1%).[3] SM4s, Wistar rat, 84-Day-old, cerebrum, 4 μ mol/g;[88] 90-day-old, spinal cord, 11 μ mol/g;[88] 175 day, brain, 3.7 μ mol/g; 310 day 4.9 μ mol/g.[227] Rat (14 day), cerebrum, SM4s (nonhydroxy fatty acids), 120 nmol/g; SM4s (hydroxy fatty acids) 63 nmol/g; cerebellum, SM4s (nonhydroxy fatty acids), 244 nmol/g; SM4s (hydroxy fatty acids); 112 nmol/g; brain stem, SM4s (nonhydroxy fatty acids), 724 nmol/g; SM4s (hydroxy fatty acids), 285 nmol/g.[431] SM4s + SM4g, Wistar rat, 6.8 μ mol/g; chronically diazepam treated, 1.5 μ mol/g.[621] SM4s + SM4g, 8-months-old SD rat, cerebellum, control, 1.08 μ mol/cerebellum; alcohol-fed, 0.25 μ mol/cerebellum.[620] 8-months-old Wistar rat, brain, 6.8–6.9 μ mol/g; fed ethanol and protein-deficient diet for 6 months, 5.3 μ mol/g; 3 months after switching to a high protein diet, 8.4 μ mol/g[619]

SM4s, human brain, frontal lobe, white matter, 12.0 μ mol/g; frontal lobe, gray matter, 2.2 μ mol/g.[420] Frontal lobe (1.7–11 y), 2.2–6.0 μ mol/g; frontal lobe, metachromatic leukodystrophy, 8.2–13.3 μ mol/g.[477] Human brain, white matter, 8.2 μ mol/g.[441] White matter, HPLC using a Nucleosil column, control, 5.1 weight%; alcoholics, 4.6%.[445] SM4s (d18:1/24:1, 24 h:0), bovine brain, (+).[430] SM4s, bear, cerebrum, 6.9 μ mol/g; cerebellum, 5.3 μ mol/g[499]

SM4s, mouse gliomas, 300 nmol/10^9 cells.[85] Various neural tumors of rat brain, 0–8.4% of lipid weight.[72] C6 glioma cell treated with disipramine, (+).[592] TLC-OL, (+), glioblastoma of grade IV; (–), glioblastoma multiforme, astrocytoma grade II, anaplastic astrocytoma.[244] Oligodendrocytes, [^{35}S]-labeling, (+).[27,111] Human oligodendroglioma, immunohistochemistry, (+).[543] SM4s [^{14}C-stearic acid], chemical preparation[96]

SM4g (A_2Gro + EAGro forms), mouse cerebellum, 28-day-old, 900 nmol/g; 14 days, 42% of the total cerebellar sulfatides.[52] SM4g (A_2Gro form), rat brain, [^{35}S]-labeling, (+).[120] SM4g (A_2Gro + EAGro forms), mouse (23-day-old), brain, 490 nmol/g; non-myelinating jimpy mouse, 69 nmol/g; Wistar rat (body weight 300 g), brain, 190 nmol/g.[343] SD rat brain, before 10 days of age, (–); between 10–25 days, 0.3–0.4 μ mol/brain; rat large myelin fraction 13.6 μ mol/g (136 nmol/mg protein).[452] SM4g, Wistar rat, brain, 48 days, A_2Gro form, 50 nmol/brain; EAGro form, 180 nmol/brain; A_2Gro + EAGro forms, 210 nmol/brain; 175 days (EAGro form), 20 nmol/brain; 175 days (A_2Gro form), 1.5 nmol/brain (total 21 nmol); 310 days, SM4g (A_2Gro + EAGro forms), not detected.[227,228] SM4g (EAGro form), 84-day-old Wistar rat, spinal cord, 600 nmol/g.[88] Wistar rat (17-day-old), central nervous system myelin, [^{35}S]sulfate incorporation, 16% of the total sulfatides[226]

SM4g, rabbit brain, approx. 50 nmol/g; human brain frontal lobe, (–).[343] Adult human brain, (–); brain, metachromatic leukodystrophy patient, (–)[228]

Lyso-SM4s, normal human frontal lobe, 50–180 nmol/g; frontal lobe, metachromatic leukodystrophy, 9–45 nmol/g.[477] Lyso-SM4s (d18:1), chemical preparation[125,569,616]

SM4s-6(d18:1/24:0) (GalCer I^6-sulfate), chemical synthesis[364,430,572]

SMUnLc$_4$Cer + SMUnLc$_6$Cer, mouse cerebellum, 400–600 nmol/g (4–6 ng/mg protein).[66] Cerebellum, murine mutants lurcher (*Lc*/+) and (*pcd/pcd*) (Purkinje cell degeneration), staggerer (*sg/sg*) (Purkinje cell defect), (–); *nr/nr* mutant, approx. 70% reduction[65]

SMUnLc$_4$Cer, rat (embryonic day 19), cerebral cortex, 1.4 nmol/g (11.2 μg/g dry tissue).[67] SMUnLc$_4$Cer + SMUnLc$_6$Cer, rat forebrain, embryonic stage, (+); soon after birth, (–).[381] Immunohistochemistry, growth corn of embryonal rat brain, neuronal cell bodies.[44] SMUnLc$_4$Cer (d18:1/16:0,18:0 and 18:1), rat cerebellum, (+).[67] SMUnLc$_4$Cer, rat cerebral cortex, microvessel fraction, 4.8–6.6 nmol/g (76–106 ng/mg protein); cultured endothelial cells, 3.2 nmol/g (51 ng/mg protein).[379] BMEC (cultured bovine brain microvascular endothelial cells), 7-day-culture, 4.1 nmol/g (65 ng/mg protein); 14-day-culture, 1.3 nmol/g (21 ng/mg protein)[261]

SMUnLc$_4$Cer, human fetal (37 week) brain, 0.94 nmol/g (7.5 μg/g dry tissue); cortex (adult), < 0.1 μg/g dry tissue; cerebellum, adult, 1.6 nmol/g (12.5 μg/g dry tissue)[67]

Peripheral nervous tissue, Schwann cell and cerebrospinal fluid

HSO_3-Chol, rat (43-day-old), adrenals, 22–30 nmol/g (51–69 μg/g dry weight).[243] Bovine adrenals, 3.2 nmol/g (1.5 μg/g); human adrenal tumor (Cushing's syndrome), 86–430 nmol/g (40–200 μg/g).[94] Schwann cell line D6P2T, (–).[26] G361 human melanoma cells, trace[469]

SM4s, adult Wistar rat, superior cervical ganglia, 0.06 mol/mol phospholipid; nodose ganglia, 0.09; SM4s (d18:1/24h:0), vagus fibers, 0.12; SM4s (d18:1/24:0, 24 h:0), dorsal root ganglia, 0.14.[178] Dog, sciatic nerve, 70 nmol/g.[416] SM4s (d18:1/24:0, 24:1, 24 h:0), human femoral nerve, 3 μ mol/g[551]

SM4s, human peripheral nervous system (PNS) myelin-1, motor, 1.9% of the total lipid; sensory, 2.5%; PNS myelin-2, motor, 1.1%; sensory, 1.4%.[434] Human cauda equina, 10 μ mol/g.[553] Human cauda equina, myelin, 62 mg/g dry myelin.[553] Human spinal cord, 11 μ mol/g tissue; human spinal cord myelin, 39 mg/g dry myelin.[553] Human motor nerve myelin, 53 nmol/g; motor nerve axons, 12 nmol/g; sensory nerve myelin, 49 nmol/g; sensory nerve axons, 12 nmol/g.[552] SM4s (d18:1/24:0, 24:1, 24 h:0), human skeletal muscle, 1 nmol/g.[551] Schwann cell line D6P2T, (+).[26] SM4s (hydroxy fatty acids), Schwann cell line S16 and S42, immunostaining, (+).[594] Schwann cell line S16, [^{35}S]incorporation, (+).[110] SM4s, mouse, G26-20 cells (one of the G26 series glioblastoma cell lines) 300 nmol/10^9 cells.[85] Melanoma cell lines G361, and C32, [^{35}S]labeling, (+).[469] The globular carboxyl domain of laminin A chain, a melanoma cell line A2058, (+).[583] A melanoma cell line, TLC-OL, (+)[509]

SM4s, human cerebrospinal fluid, 140 nmol/l[84]; control 145 ± 86 nmol/l; vascular dementia 307 + 118 nmol/l; Alzheimer, 178 ± 79 nmol/l.[122,124] Normal control, 99 nmol/l; AIDS patient, 395 nmol/l[142]

SM4g, Schwann cell line D6P2T, (+).[26] Melanoma cell lines G361, and C32 (by [^{35}S]labeling), (–)[469]

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Table 3—*continued*

SM3, melanoma cell lines G361, and C32, [^{35}S]labeling and TLC autoradiography, (+);[469] a melanoma cell line A2058, (+)[583]

SMUnLc$_4$Cer, dog sciatic nerve, 4.5 nmol/g; SMUnLc$_6$Cer, 3.3 nmol/g[416]

SMUnLc$_4$Cer, human spinal cord, 1.8 nmol/g; human, cauda equina, 64 nmol/g; femoral nerve, 39 nmol/g; cauda equina myelin, 112 nmol/g.[552] Human, sciatic nerve, adult, 8.2 nmol/g (65.0 μg/g dry tissue);[67] 53 nmol/g (0.85 μg/mg protein).[16] Human cauda equina, 99.7 nmol/g (1.59 μg/mg protein).[16] Adult human, motor (ventral root) nerve, 54 nmol/g; sensory (dorsal root) nerve, 72 nmol/g; motor nerve myelin, 193 nmol/g; motor nerve axons, 70 nmol/g; sensory nerve myelin, 199 nmol/g; sensory nerve axons, 80 nmol/g.[552] Schwann cells, (+)[249]

SMUnLc$_6$Cer, rat, sciatic nerve, adult, 0.44 nmol/g (3.5 μg/g dry tissue)[67]

SMUnLc$_6$Cer, human spinal cord, trace; cauda equina, 15 nmol/g; femoral nerve, 10 nmol/g; motor nerve, 16 nmol/g; sensory nerve, 20 nmol/g; motor nerve myelin, 63 nmol/g; motor nerve axons, 25 nmol/g; sensory nerve myelin, 72 nmol/g; sensory nerve axons, 28 nmol/g[552]

SMUnLc$_4$Cer (SMUnLc$_6$Cer, human dorsal root ganglia, 64 nmol/g (1.02 μg/mg protein); human sympathetic ganglia, 2.5 nmol/g (0.04 μg/mg protein); dorsal root light myelin, 94 nmol/g (1.50 μg/mg protein); heavy myelin, 99 nmol/g (1.57 μg/mg protein); axolemma-enriched fraction, 107 nmol/g (1.71 μg/mg protein)[15,16]

SMUnLc$_4$Cer + SMUnLc$_6$Cer, melanoma G361 cells, [^{35}S]labeling, and TLC-OL with HNK-1 antibody, (+)[469]

Blood cells, serum, blood vessels and spleen

HSO$_3$-Chol, human red blood cells, 5.0 nmol/g; human plasma, 3.1–3.9 nmol/ml.[41] Serum, X-linked ichthyosis patient, elevated.[661] Human plasma, GC, normal control, 5.4 nmol/ml (253 μg/dl); cirrhosis patient, 9.6 nmol/ml (445 μg/dl); hypercholesterolemia patient, 8.9 nmol/ml (414 μg/dl).[580] Human (> 40-y), aorta (intima + media), 32 nmol/g (15 μg/g)[94]

HSO$_3$-Chol, rat spleen, 33 nmol/g (77 μg/g dry tissue); thymus 1.8–1.9 nmol/g (4.2–4.5 μg/g dry weight).[243] Rat spleen, [^{35}S]-labeling, (+)[354]

SM4s (d18:1/16h:0), human erythrocytes, 0.62 nmol/g red blood cells (3.3 mg/6.7 kg wet cells).[175] Sheep, packed red blood cells, 5.4 nmol/g (4.3 mg/kg packed erythrocytes).[471] SM4s (d18:0, d18:1/16h:0), a bovine erythrocyte membrane preparation, 0.5 nmol/g (0.37 μg/g)[323]

SM4s, human platelets, 1.5 nmol/g.[471] SM4s (d18:1/24:1 and 16 h:0), human, platelets, (+).[394] SM4s, human granulocytes, flow cytometry and fluorescence microscopy, cell surface, (+); excreted into the medium, 5 μg/10^8 cells/12 h; (+) breast carcinoma and myeloid cell lines; (–), leukemic T cell lines, HeLa and COS cells.[17] Leukocytes, immunodetection using the monoclonal antibody Sulf-I, (+).[56] SM4s (d18:1/24:0), bovine spleen, 1.1 nmol/g(1 μg/g tissue)[496]

SM4s, porcine plasma,^{1}H-NMR, (+).[165] SM4s (d18:1/22:0), mammalian sera: pig, 16 nmol/ml; goat, 13 nmol/ml; cow, 9 nmol/ml; horse, 8 nmol/ml; sheep 1.2 nmol/ml; human, 0.64 nmol/ml; dog 0.16 nmol/g; rat, mouse, chicken, not detected.[653] SM4s, normal rabbit, serum, 3 nmol/ml; WHHL rabbit, serum, 121 nmol/ml; chylomicron, 1.2 mol% of phospholipids; VLDL, 1.9 mol% of phospholipids; LDL, 1.8 mol% of phospholipids; HDL, 1.4 mol% of phospholipids.[183] SM4s, normal rabbit, aorta (–); WHHL rabbit, aorta, atherosclerotic plaque, 280 nmol/g[177]

SM3, porcine plasma,^{1}H-NMR, (+)[165]

SMUnLc$_4$Cer, T-lymphocytes, (+)[68,221]

Alimentary system

HSO$_3$-Chol, human saliva, 4.3 nmol/ml.[41] Rat stomach, [^{35}S]-labeling, (+).[354] SD rat intestine, basolateral membrane, (+ +); HSO$_3$-Chol, brush border (+).[173] Mammalian and cod fish intestine, (+).[47] Human feces, (+).[388] HSO$_3$-Chol + SM4s, human small intestinal mucosa, 5.2 mg/g dry weight[101]

SM4s (20:0) + SM3 (18:0), rat sublingual gland, 940 nmol/g; SM4s (16:0) + SM3 (16:0, 18:0), rat submaxillary gland, 310 nmol/g.[523] SM4s, rat (Wistar) glandular stomach, 28 nmol/g (136 nmol/g dry tissue).[414] SD rat small intestine, (–);[173] Wistar rat small intestine, (–) (Tadano-Aritomi, K. and Ishizuka, I. unpublished). Small intestine of hen, white rat, cat, rabbit, (+); mouse, black–white rat, guinea pig, (–).[47] SM4s, guinea pig gastric mucosa, 32 nmol/g (160 nmol/g dry tissue)[300]

SM4s (d18:1/22–24h:0), rabbit, stomach, fundic mucosa, 79 nmol/g (394 nmol/g dry tissue); antral mucosa, 71 nmol/g; duodenum, 166 nmol/g; jejunum, 76 nmol/g.[414] SM4s (t18:0/22h:0, 23 h:0, 24 h:0), rabbit intestine, 31 nmol/g; WHHL rabbit intestine, 100 nmol/g.[183] SM4s (d18:1/24h:0), ileum, 12 nmol/g (58 nmol/g dry tissue); colon, 25 nmol/g (126 nmol/g)[413]

SM4s, dog fundic mucosa, 34 nmol/g (0.17 μ mol/g dry mucosa); antral mucosa, 96 nmol/g (0.48 μ mol/g dry mucosa).[524] SM4s, dog intestine, 26 nmol/g[373]

SM4s, normal human gastric mucosa, 3.5–36 nmol/g; gastric cancer (tubular adenocarcinoma), 58–98 nmol/g.[190] Normal gastric mucosa, 56 nmol/g (0.25 μg/mg dry tissue); gastric mucosa (metaplasia), 89 nmol/g (0.40 μg/mg dry tissue); gastric carcinoma, 154 nmol/g (0.70 μg/mg dry tissue).[377] SM4s, immunohistochemistry, (+) the chief cells of the stomach.[22] Gastric cancer cell line, Kato III, (+) (a major acidic glycolipid).[259] Esophagus mucosa, 3.3 nmol/g (16 nmol/g dry weight); SM4s (d18:1/24h:0, 26 h:0), fundic mucosa, 83 nmol/g (416 nmol/g); antral mucosa, 187 nmol/g (934 nmol/g); duodenum, 137 nmol/g (683 nmol/g); jejunum, 44 nmol/g (218 nmol/g); colon, 60 nmol/g (297 nmol/g); rectum, 31 nmol/g (153 nmol/g).[412,414] Human colon (normal colonic mucosa), Azure A method using the Folch's lower phase, 20–170 nmol/g (0.20–1.70 nmol/mg protein); colonic cancer, 30–210 nmol/g (0.30–2.10 nmol/mg protein).[519]

—*continued*

Table 3—*continued*

Human colon, the upper band, 27 nmol/g, the lower band, 56 nmol/g; colorectal cancer, the upper band 65 nmol/g, the lower band 11 nmol/g.[387] Human, colon adenocarcinoma WiDr cell line, 90 nmol/g;[314] human colonic epithelial cell lines HT-29,[103] and Caco-2, (+);[103,657] Flow cytometry, Colo 205 cell, (+)[154]

SM4s (d18:1/24h:0), porcine gastric mucosa, 60 nmol/g.[526] SM4s (22 h:0), pig intestine, 11 nmol/g (2 mg/22 kg)[548]

SM1b, C57B1/J mouse intestine, (+);[47] 7-week-old male, intestine, approx. 50 nmol/g (Tadano-Aritomi, K, and Ishizuka, I, unpublished).

Liver, gallbladder, and pancreas

HSO_3-Chol, rat (43-day-old) liver, 7–9 nmol/g (16.4–20.2 μg/g dry tissue).[243] Human liver, 130 nmol/g (60 μg/g); human gallstones, 1.1 μ mol/g (500 μg/g);[94] metachromatic leukodystrophy patient, 3.5 y, liver, 130 nmol/g.[388] A patient with GM1-gangliosidosis, liver, 225 nmol/g[182]

SM4s, mouse liver, [^{35}S]label and silicic acid column, 416 nmol/g; rat liver, 22 days of age, 478 nmol/g; 117 day, 213 nmol/g;[24] CCl_4-induced liver inflammation (+).[252] SM4s, rabbit liver, 416 nmol/g.[23] SM4s (d18:1/22:0, 23:0, 24:0), rabbit, liver, 104 nmol/g; rabbit (WHHL), liver, 260 nmol/g.[183] SM4s (d18:1/24:0), rabbit hepatocyte plasma membrane, (+)[593]

SM4s, normal human liver, < 20 μg/g dry tissue.[539] SM4s (d18:1/24h:0), metachromatic leucodystrophy (MLD) patient, liver, 62 nmol/g (280 μg/g dry tissue).[539] Normal human liver, (+); liver, a patient of atypical Farber disease, (+ + +).[131] SM4s (24:0), metachromatic leucodystrophy patient, liver, 3% of the total lipid; gallbladder, SM4s (d18:1/24:0), 35%.[2] SM4s (d18:1, d18:0/24h:0), a patient with GM1-gangliosidosis, liver, 69 nmol/g[182]

SM4s, [^{35}S]labeling/TLC, (+), a human hepatocellular carcinoma cell line PLC/PRF/5; a human cultured cholangiocarcinoma cell line, H-1; HepG2, KYN-1, KMCH-1 cell lines derived from hepatocellular carcinoma[204]

SM3, liver, normal human, (–) (< 20 μg/g dry tissue); SM3 (d18:1/24h:0), metachromatic leukodystrophy, 39 nmol/g (208 μg/g dry tissue).[539] SM3 (18:1), metachromatic leukodystrophy, 2% of the total lipid; gallbladder, 6%[2]

SM3, [^{35}S]labeling/TLC, (+), human hepatocellular carcinoma cell lines PLC/PRF/5, HepG2, KYN-1, KMCH-1[204]

SB1a, [^{35}S]labeling/TLC, (+), a human hepatocellular carcinoma cell line PLC/PRF/5[204]

SM4s (d18:1/16:0 and 18:0), porcine pancreas, 10% by weight of the total acidic glycolipids.[407] SM4s, human pancreas, immunological detection using monoclonal antibody Sulf I, 84 nmol/g; Langerhans islet, 150 pmol/100 islets (approx. 8.4 μ mol/g islet tissue).[56] Lewis rat islet, 410 pmol/100 islets;[56] BB rat islet 87 pmol/100 islets; Wistar rat islet 181 pmol/100 islets; Lewis rat B cells 13 pmol/10^5 cells[56]

Male reproductive organs

HSO_3-Chol, rat (43-day-old), testis, 15 nmol/g (34 μg/dry tissue).[243] Rabbit treated with estradiol, testis, trace; prostate, 33 nmol/g (0.33 nmol/mg protein).[81] Human semen, 14 nmol/ml,[41] human spermatozoa (acrosome), (+).[331] Human seminal plasma 9.6 nmol/ml (445 μg/100 ml); sperm 32 nmol/10^9 cells (15 μg/10^9 cells).[329,473] Rhesus monkey, sperm, 64 nmol/10^9 cells (29.8 μg/10^9 cells).[348] Boar sperm plasma membrane, caput epididymidis, 0.8 nmol/10^9 sperm; corpus, 0.6 nmol; cauda 1.4 nmol.[428] Boar sperm acrosomal membrane, 140 nmol/g (1.4 nmol/mg protein).[429] Boar sperm plasma membrane, before acrosome reaction, 410 nmol/g (4.1 nmol/mg protein); 1 hr after acrosome reaction, 40 nmol/g (0.4 nmol/mg)[427]

Desmosteryl sulfate, hamster epididymis, the major sterol sulfate.[473] In rhesus monkey sperm not sulfated[348]

SM4g ($E_{16:0}A_{16:0}$), C57 mouse, 800 nmol/g; W/W^{v}mutant, 120 nmol/g.[306] Wistar rat, 7- to 10-day-old, testis, 100 nmol/g (1 nmol/mg protein); 30-day, 1400 nmol/g (14 nmol/mg protein); hypophysectomized rat, reduction of the SM4g concentration to 70% of the control.[306] Mature rat, 2275 nmol/g testis (1.9 mg/g).[119] SD rat testis, Azure A assay of the Folch lower phase lipids, 400 nmol/g, plasma membrane fraction, 13.5 μ mol/g.[517] Rat, fed on vitamin A supplemented chow for 46 days, 556 nmol/g; fed on vitamin A deficient chow, 73 ± 46 nmol/g;[546] Lewis rat, 4.9 μ mol/g.[56] Rat (23 day) whole testis, 800 nmol/g (8 nmol/mg protein); rat spermatocyte, 4.4 μ mol/g (44 nmol/mg protein).[341] Guinea pig, testis, 437 nmol/g.[545] Rabbit treated with estradiol, testis, 400 nmol/g (0.40 nmol/mg protein)[81]

SM4g ($E_{16:0}A_{16:0}$), boar testis, about 300 nmol/g (0.8% by weight of the total lipid); boar spermatozoa, 1040 nmol/g cells.[232] Boar spermatozoa, perbenzoylation/HPLC, 980 nmol/g.[544] Boar spermatozoa, 1.4 μ mol/g cells.[134] SM4g plasma membrane of boar sperm obtained in epididymal caput, acetone fraction from silicic acid determined by Azure A method, 38.3 nmol/10^9 sperm; corpus, 26.9 nmol; cauda, 19.6 nmol;[428] boar sperm plasma membrane, 4.6–5.9 μ mol/g;[428] acrosomal membrane, 4.6 μ mol/g (46 nmol/mg protein).[427,429] Bull sperm, 8% of the total lipid[511]

SM4g, human (2 m–11 y), (–).[342] SM4g ($E_{16:0}A_{16:0}$), human (40–50 y) testis, 170 nmol/g; seminoma, (–);[238] 2–9 y, (–); 40 y, 159 nmol/g; 60–90 y, 25 nmol/g[605]

SM4g ($E_{16:0}A_{16:0}$), chemical synthesis[141]

Female reproductive organs

HSO_3-Chol, rat (43-day-old) uterus, 4.9 nmol/g (11.5 μg/g dry weight); ovaries 14.8 nmol/g (34.3 μg/g dry weight).[243] Rabbit, endometrium, normal, 40 nmol/g; pseudopregnancy (day 4), 380 nmol/g[384]

SM4s (d18:1/24h:0), human, uterine endometrium, proliferative (follicular) phase, 1–3 nmol/g (7–17 nmol/g dry tissue); secretory (luteal) phase, 23–49 nmol/g (115–245 nmol/g dry weight);[318] secretory phase, 12–46 nmol/g (60–230 nmol/g dry tissue).[319] Human, a uterine endometrial adenocarcinoma, 62 nmol/g (trace to 310 nmol/g dry tissue).[319] Human endometrial adenocarcinoma cell line Ishikawa, [^{35}S]sulfate incorporation, (+)[255]

—*continued*

Table 3—*continued*

SM4s, Lewis rat, ovary, 7 nmol/g.[56] SM4s, normal human ovary, (–); SM4s (t18:0/22:0) mucinous cystadenocarcinoma (a clear cell adenocarcinoma), TLC-densitometry, 230 nmol/g (1.14 μ mol/g dry weight) (> 90% of acidic glycolipids).[285] Choriocarcinoma cell line Rcho-1 (rat), TLC, (+).[516] Human amnion, 338 pmol/g[18]
SM3, human, uterine endometrium, secretory phase, 8 nmol/g (40 nmol/g dry tissue); SM3 (d18:1/24:0), human, a uterine endometrial adenocarcinoma, FAB, 46 nmol/g (trace to 230 nmol/g); SNG-II cell line derived from human endometrial adenocarcinoma, 158 nmol/g (790 nmol/g dry weight).[319] Human endometrial adenocarcinoma cell line HEC 108, [^{35}S]sulfate incorporation, (+).[255] Human ovarial clear cell carcinoma, (+).[285] Human amnion, 108 pmol/g[18]
SM2a (d18:1/24:0), human, a uterine endometrial adenocarcinoma, FAB, (+); SNG-II cell line derived from human endometrial adenocarcinoma, 60 nmol/g (300 nmol/g dry weight).[319] Human endometrial adenocarcinoma cell line HEC 108, SNG-M and SNG-II, [^{35}S]sulfate incorporation, (+)[255]
A sulfo-Gg_4Cer, human, a uterine endometrial adenocarcinoma, (+)[319]

Weights on dry weight or protein basis were recalculated into μ mol/g or nmol/g fresh tissue using the molecular weight of the major molecular species assuming the tissue water and protein content of 80 and 10% respectively unless otherwise specified.

cell respectively, supporting the results obtained by chemical analyses. The fatty acid composition of SM4s in human muscles and femoral nerve was very similar suggesting that SM4s of skeletal muscles was predominantly derived from the nerves in the muscle.[551]

Guillain-Barré syndrome (GBS) has been characterized as an acute inflammatory demyelinating polyneuropathy, in which myelin is assumed to be the target of immune attack. The sera from patients with GBS displayed anti-SM4s antibodies in 67% of cases.[122,123] Serum antibodies from infants with congenital HCMV (human cytomegalovirus) infection interacted on TLC with the component sulfatides of the peripheral nerve, SMUnLc$_6$Cer and SMUnLc$_4$Cer, with high affinities, and with SM4g, SM4s, and SM3 with low affinities.[435] Furthermore, the serum IgM from the HCMV infected patient interacted with a variety of sulfated glycolipids including SM4g, SM3, but not to HSO_3-Chol, suggesting that the sulfated sugar chains were the epitopes for the low-affinity interaction.[435] Two glycolipids, SMUnLc$_4$Cer and SMUnLc$_6$Cer, from human peripheral nerve and cauda equina, react with monoclonal antibody HNK-1[66,70] and the IgM in patients with paraproteinemia and peripheral neuropathy.[221,656] They are present in human, monkey, bovine, dog, and cat peripheral nerves or in greatly reduced amounts in rat, mouse, rabbit, guinea pig and chicken.[221,249]

3. *Blood, Blood Vessels, and Spleen*

Red blood cells (RBC), white blood cells, blood plasma, thymus, and spleen contain low concentrations of HSO_3-Chol and/or sulfatides (Table 3).

SM4s is one of the major glycolipids in serum lipoproteins of various mammals except rodents[183,653] and enriched in the atherosclerotic plaque of Watanabe Hereditary Hyperlipidemia (WHHL) rabbit.[177] In serum lipoprotein of WHHL rabbit, an animal model for human familial hypercholesterolemia, SM4s content was elevated by about 40-fold (120 nmol/ml) (Table 3) over the normal level.[183] The intravenous administration of SM4s to WHHL rabbits lowered the level of triacylglycerols in serum, suggesting that SM4s might activate lipoprotein lipase or hepatic triacylglycerol lipase.[571]

Negatively charged surfaces, phosphatidylinositol phosphate, SM4s, and dextran sulfate autoactivated the plasma contact activation system including human factor XII.[475] Unlike heparin, SM4s failed to inhibit thrombin and coagulation factor Xa activities in the presence of antithrombin III (AT III). The SM4s micelle rather bound specifically to fibrinogen (400 mol SM4s per fibrinogen molecule) and thereby may interfere with both fibrin gel formation (anticoagulant activity) and platelet function[179] suggesting that SM4s may be an effective endogenous component for the prevention of thrombosis.[184] Actually, exogenous SM4s added to rabbit serum to 8 nmol/ml prolonged the fibrin-pre-

cipitation time.[653] HSO_3-Chol showed no anticoagulant activity, while SM4s-6 was 20 times more potent anticoagulant, although the latter produced antibodies. Since AIDS drugs must be administered into the blood stream (cf. VII. D), the anticoagulant activity of sulfated polysaccharides may result in a significant side effect.[276,277]

Staining with Sulf-I monoclonal antibody indicated that SM4s was expressed on the plasma membrane of granulocytes.[17,56] P-selectin[17] interacted with SM4s on the cell surface of granulocytes and several myeloid tumor cells. Exogenous SM4s may stimulate various cellular activities including the oxygen radical production of leukocytes (0.5×10^{-4}–7×10^{-4} M)[64,253] or neutrophils ($> 10^{-5}$ M);[30,335] cytosolic Ca^{2+} level and oxidative burst of neutrophils via tyrosine kinase/MAP kinase system ($> 5 \times 10^{-5}$ M);[30,622] monocyte phagocytosis of sulfatide-enriched human erythrocytes mediated possibly by thrombospondins;[513] accumulation of Ca^{2+}, and secretion of interleukins with enhanced production of related mRNA in monocytes (4×10^{-4} M).[77] On the other hand, SM4s coated on *Staphylococcus aureus* cells stimulated phagocytosis of neutrophils, while had not effects on the superoxide anion release.[635] Exogenous HSO_3-Chol was a better stimulant than SM4s, while SM4g, SM4s-6 and glucose 6-sulfate did not significantly stimulate the oxygen radical production of leukocytes.[253] SM4s specifically triggered the increase of cytosolic free Ca^{2+} in neutrophils most probably through interaction with L-selectin.[30,335] Secretion of TNF-α and IL-8 in monocytes (400 μg/ml),[77] and neutrophils (> 10 μg/ml)[335] by SM4s, may correspond to the similar reaction elicited by 100 ng/ml of LPS. Currently drugs are under development to interfere with the interaction of selectins with their ligands. They include monoclonal antibodies to block the adhesive molecules, or competitive ligand saccharides, e.g. SM4s,[393] 3′-sulfo-Lewis x, 3′-sulfo-Lewis a,[613] and lactose 6,6′-disulfate.[33] Not only vertebrate sulfatides, but also other acidic glycolipids including gangliosides, the monosulfated cord factor or TDM (trehalose dimycolate), and GlcU-containing sphingolipids from bacteria stimulated adhesion, phagocytosis, and phagosome-lysosome fusion of human neutrophils.[380]

4. *Alimentary System and Liver*

The mammalian saliva,[41] gastrointestinal tract[47,101,242] and feces[388] contained HSO_3-Chol. The sublingual and submaxillary glands, and the gastrointestinal tract of mammals contained sulfatides[81,361,537] (Table 3), whereas human saliva and gastric juice did not.[411] The keratinizing squamous epithelium lining of the esophagus concomitantly showed high expression of prosaposin mRNA in the basal and spinous layers probably related to processing of sulfated lipids.[540] The highest concentration of SM4s in the gastrointestinal tract was found with antral or duodenal mucosa of humans.[412] In contrast, the concentrations of lipid-bound sialic acid in gastric mucosa, especially of fundic mucosa, were strongly reduced than those in the other parts of gastrointestinal tract[524] resulting in the SM4s to lipid-bound sialic acid ratio of 1.4:2.3.[412]

Sulfatides were isolated also from the intestine of mammals including porcine,[548] dog,[373] mouse,[339] and WHHL rabbit,[183] and SM4s was even a major glycolipid for cat, rabbit, guinea pig, and hen intestine, reminiscent of the common developmental origin of intestinal epithelia and renal tubular cells.[47] Exceptionally mouse, rat and cod intestine did not contain SM4s, possibly due to the lack of GalCer similarly to some glioblastomas.[244] Instead, HSO_3-Chol was enriched in the basolateral membrane of the rat small intestine, suggesting that HSO_3-Chol can replace SM4s.[173] Analogously, it appeared an opportunistic strategy for mice (C57Bl/J strain) to sulfate Gg_4Cer in epithelial cells of small intestine, which lacks GalCer, to produce SM1b constituting at least 90% of the acidic glycolipids.[339]

Mucosal localization of SM4s has been demonstrated in the gastrointestinal tract of porcine,[526] guinea pig,[300] dog,[524] rabbit,[413,651] and human.[101,190,377,412,519] The glycolipids were enriched in the epithelial but not in the nonepithelial compartment of the rat large intestine.[173] Both surface epithelial and glandular (parietal and chief) cells in the gastric mucosa of rabbit and human, but not the secretory granules, contained SM4s as

shown by immunofluorescence staining using a monoclonal antibody.[538] It has been deduced that intestinal cell glycolipids make up even one third of the lipids in the apical membrane[657] and essentially cover the whole surface of the luminal leaflet. The pre-epithelial mucosal defense activities[78] together with surface epithelial negative charges[538] may protect mucosal cells against autodigestion by acid and pepsin, similarly to sucralfat (sucrose polysulfate aluminum complex), which has been widely used for treatment of gastritis and gastric ulcer.[414]

[^{35}S]-labeling showed the presence of SM4s in rat liver,[24,151] although sulfatides were not detectable by chemical methods in the liver, except for the rabbit.[183] In the liver of patients of metachromatic leukodystrophy[2,539] and in a patient of atypical Farber disease,[131] SM4s and SM3 were accumulated. Sera from patients with autoimmune chronic active hepatitis[593] and systemic lupus erythematosus[14] contained an IgG-class antibody to both the acidic glycosphingolipid fraction from rabbit hepatocyte plasma membrane, and SM4s. The reactivity of the hepatitis serum with SM4s was diminished by preincubation of the serum with SM4s-6 and SM4s itself, indicating that the antibody reacted with sulfated GalCer regardless of the position of the sulfate residue.

Sulf I monoclonal antibody immunogold staining showed the secretory granules of both A and B cells of the rat Langerhans islet contained SM4s. Furthermore, the SM4s synthesis was entirely abolished by destroying islets by streptozotocin treatment.[56,57] Assuming that 1% of the tissue weight of a pancreas consists of islets, SM4s concentration in human islets should be 8.4 μmol/g islet tissue, which is about one third of the concentration in the white matter of the human brain.[56,57] SM4s antibody may be an IDDM (insulin dependent diabetes mellitus) marker because sera from 88% of newly diagnosed IDDM patients were anti-SM4s positive, and 76% were positive 6 months later while all healthy controls were negative.[55] IDDM patients' sera positive for SM4s stained secretory granules in A and B cells of rat islets of Langerhans.

5. Reproductive Organs

HSO_3-Chol was enriched at the plasma membrane of human,[331] and boar[427,428] spermatozoa and acrosomal membrane of boar.[427] HSO_3-Chol or other steroid sulfates (HSO_3-Chol + desmosterol sulfate) of hamster[473] and boar[428,429] spermatozoa increased during their epididymal transit. In contrast, the SM4g content of the sperm plasma membranes decreased by about 50% during epididymal transit from the caput to the cauda.[428] Then, at capacitation in the female reproductive tract, drastic desulfation of HSO_3-Chol was observed at the plasma membrane of hamster.[331,473] Steroid sulfates, including HSO_3-Chol, have been known to stabilize certain membranes like the erythrocyte plasma membrane[107,329] and keratinocytes.[107,630]

In the testis of mammals including rat,[238,395] guinea pig,[545] human,[237,342,606] boar,[232] and bovine,[354] SM4g (seminolipid) is the principal glycolipid of testis comparable in amount with HSO_3-Chol[348] (w/w). In the lipid from ejaculated boar spermatozoa, SM4g comprised approx. 3% of the total lipid, that is more than 90% of the sum of glycolipids, and the concentration (1 μmol/g wet cells) was approximately three times greater than that in the testis.[232] The w/w^v mouse testis, with a greatly reduced content of all germinal cells, contained a negligible amount of SM4g.[306]

Between 11 and 20 days in mice, intraperitoneally injected [^{35}S]sulfate was actively incorporated into SM4g of the testis.[171,233] There were only low amounts of lipid-bound galactose and lipid-bound sulfate in the testis of rats younger than 15 days,[306] whereas between 15–22 days in rat,[306] there was a dramatic increase in both lipid-bound sulfate and a sulfotransferase activity, although the total lipid concentration remained relatively constant.[395] This age corresponds to the appearance of spermatocytes in rat testes and actually, isolated late spermatocytes contained SM4g at 5 times the level in the whole testis.[341] The activity of GalCer sulfotransferase in rat testes increased in maturing rats of 10–15 days, then reached a plateau from day 15–26 after birth, and finally decreased to one third of the maximal activity in rats of 50 days or older.[305,341,517] [^{35}S]sulfate,[545] [2-

^{14}C]Gro, and dihydroxy[U- ^{14}C]acetone[511] were not incorporated into SM4g of spermatozoa, indicating that no synthesis of SM4g occurred after ejaculation. On day 28 after the pulse-labeling of rat with [^{35}S]sulfate, the majority of total radioactivities of the testicular lipid [^{35}S] declined and reached a minimum level on day 34.[341] Conversely, the radioactive level in the caudal epididymal sperm reached a maximum on day 32. Specific activities of the radiolabel in both the epididymides and the testes followed a similar trend. The highest specific activity on day 32 in the epididymis was up to 180 times greater than that in the testis.[582]

Neither SM4g nor Gal-EAGro was detected in the testes of infants or a child of prepubertal age.[606] At 40 years, both the total lipid and SM4g contents were highest.[606] Above 70 years of age, the concentration of SM4g decreased drastically, to approx. 1/6 of that in the 40 s, probably due to the reduction in the number of seminal tubules. In contrast, ganglioside content was highest in the testis of the aged, probably reflecting the fibrosis of the testis.

By using a monoclonal antibody specific to sulfatides, a dense staining inside each seminal tubule was noted in the rat testis, and in epididymis an intense labeling of the spermatozoa was seen.[56] Monoclonal antibodies specific to SM4g also showed that SM4g was enriched at the plasma membrane of spermatozoa.[97,135,428,447,517] Actually, the isolated boar sperm acrosomal membrane contained an extremely high concentration of SM4g.[428] In freshly ejaculated sperm cells, SM4g was present primarily at the apical ridge subdomain of the plasma membrane of the sperm head.[135] During capacitation of the sperm cells by Ca^{2+} ion,[643] SM4g migrated rapidly from the apical ridge to the equatorial subdomain of the plasma membrane.[135] An addition of arylsulfatase A to the sperm cells desulfated SM4g, and the product Galβ-EAGro also appeared specifically at the equatorial ridge. The interaction and degradation of the sperm SM4g by *M. pulmonis* may play a role in the induction of infertility that follows infection with these organisms by interfering in sperm/egg receptor recognition.[354] Prosaposins are contained in the seminal plasma[202] and the substrate glycolipids are located in, or at the exterior surface of the plasma membrane and acrosomal membrane of spermatozoa.[134,135,618]

The presence of arylsulphatase A and SM4s in different tracts of *Rana esculenta* oviduct during different phases of the reproductive cycle was studied by histochemical and biochemical procedures.[615] The results indicate that enzyme activity and SM4s level show seasonal fluctuations connected with the phase of the sexual cycle. The SM4s concentration in human endometrium also changed associated with the menstrual cycle. The concentration at the proliferative phase, 1–3 nmol/g, was elevated to 23–49 nmol/g at the secretory (luteal) phase[254,318,319] concomitant with the elevation of galactosyl-[255] and sulfotransferase activities.[254] In rabbit,[384] and guinea-pig,[422] which are deficient in glandular organization, sulfatides were present in very small quantities in endometrium irrespective of the reproductive stage, whereas HSO_3-Chol increased sharply at day 5 of pregnancy, which is the beginning of implantation.[384] Pseudopregnancy of rabbit or administration of human chorionic gonadtropin after estrogen priming also stimulated HSO_3-Chol synthesis and in the latter case, 15% of the total cholesterol was converted to the sulfated form concomitant with a 30-fold increase of cholesterol sulfotransferase activity.[384] As in the rat intestine (V.C.4), HSO_3-Chol in the endometra of these animals appears to replace SM4s. In the rat ovary, Sulph I (an anti-SM4s monoclonal antibody) distinctly labeled the ovum (oocytes), whereas the follicle epithelium, the theca and rest of the ovary were unstained.[56] In the rat oviduct (uterine tube), parts of the epithelium were labeled, whereas the uterus was negative.

6. *Tumors*

Sulfatides are expressed in mammalian malignancies.[121,137] For instance, sulfatides were increased in the adenocarcinomas including gastric[190,377] and renal cell carcinoma (Grawitz),[490] but not in Wilms' tumor tissue.[489] The accumulation of sulfatides was associated with the elevated activity of sulfotransferase in renal cell carcinoma,[490] and gas-

tric adenocarcinoma.[351,377,489] Human adenocarcinoma tissues, originated from the gastrointestinal tract,[387,519] and the related cell lines[314] also contained high concentrations of sulfatides (Table 3). It is interesting that the level of GalCer sulfotransferase was the highest in intestinal metaplasia.

In contrast to the differentiated, thus only weakly tumorigenic renal epithelial cell lines (MDCK, JTC-12),[555] the specific activity of sulfotransferase toward GalCer in the renal cancer cell line SMKT-R3[293] was 50-fold greater than that in normal human kidney tissue (42.0 pmol/mg protein/hr),[489] and 8-fold greater than that in renal cell carcinoma tissues.[293] SMKT-R3 contained SM4s, SM3, SM2a,[293] and several other minor sulfatides[211] concomitant with sulfotransferase activities synthesizing SM2b and SB2.[292] The level of SM4s in the lung of normal human adult and squamous cell carcinoma of lung was low,[645] whereas pulmonary adenocarcinoma contained more than 10 times higher SM4s than the normal lung, accompanied by an elevated activity of both GalCer sulfotransferase[645] and arylsulfatase A.[139,409]

In 29% of human hepatocellular carcinoma tissues, the accumulation of SB1a was observed.[204,205] In addition, approx. 88% of hepatoma tissues and 50% of cirrhotic liver contained a significant amount of SM3, although the level of the sulfotransferase activity in hepatoma tissues of human and rat was not distinguished from that of normal controls.[258] Clonal tumor cell lines G26-20 and G26-24 of rat, supposed to be originated from neuroectoderm, incorporated [^{35}S]sulfate into SM4s.[85] Three types of glycosphingolipid (GSL) component profiles have been established for human intracranial gliomas.[244] Only glycosphingolipid (GSL)-type III gliomas contained GalCer and SM4s.

Human testicular seminoma, an extremely undifferentiated tumor originated from the epithelium of seminiferous tubules, did not contain SM4g.[238] On the contrary, uterine endometrial carcinoma tissues contained an elevated concentration of SM4s. A high concentration of SM3, SM2a and a sulfo-Gg_4Cer appeared after 70th doubling time of the endometrial adenocarcinoma cell lines SNG-M[319] and HEC 108[255] in culture with concomitant defect of SM4s, arousing caution in the analysis of sulfatides in cultured cells. The differential expression of sulfatides between Ishikawa cells and the other three endometrial adenocarcinoma cell lines may be due to the absence of LacCer in the former and the lack of GalCer in the latter.[255] Approximately 80% of the lipid [^{35}S]sulfate in human breast ductal carcinoma T47D cells was found in HSO_3-Chol.[583]

Sulfotransferase has been implicated as the potential tumor marker enzyme. Activities of glycolipid sulfotransferase in serum were elevated in 33% of patients with hepatocellular carcinoma.[136] In comparison, the sulfotransferase levels[137] in sera from patients with renal cell carcinoma were lower than those in hepatocellular carcinoma. It remains to be clarified why hepatocellular carcinoma, which is poor in SM4s, releases more sulfotransferase into serum than does renal, pulmonary and gastric[190] carcinomas,[258] which are rich in the enzyme.

D. Molecular Evolution

The phylogenetic descendency of glycolipids has been discussed on the view point of the chemical structure of saccharides,[629] glycosyl transferase genes,[484] and Darwinian vs. neutral evolution of saccharide structure.[238] The cloning of the cDNA of A, B, and O glycosyltransferases showed that the mutation of transferase to synthesize ABO blood group antigens resulted in no pressure in selection.[640] Recently, inactivation of a specific glycosyltransferase gene is replacing the conventional 'experiments of the nature' to prepare animals with enzyme deficiencies. In many cases of deletion of a selected glycosyltransferase gene in knockout mice, the survival and the early development of the animal were apparently normal supporting the proposition that most of the oligosaccharide structures have been almost neutral in evolution.[238] However, higher and more specific functions were usually affected. In other words, oligosaccharides are analogous to the 'axle grease' of an automobile.[611] While its absence would markedly affect the ability of

the entire vehicle to function, the fine details of the composition of the grease should not be critical to the turning of the axle.

The concept of lipid class replacement by Rouser[480] explained why one of the major phospholipid, sphingomyelin, should substitute phosphatidylcholine in biomembranes. The myelin structure of mice lacking the enzyme UDP-galactose:ceramide galactosyltransferase contained GlcCer and sphingomyelin with hydroxy fatty acids instead of GalCer and SM4s.[75] The apparently compensatory distribution of HSO_3-Chol and SM4s in the gastrointestinal tract, sperm, endometrium, and kidney of various species also supports the above concept. The reversible replacement of acidic phospholipids with GlcU-containing glycolipids or SQ-A_2Gro,[155] and the polysaccharide teichoic acid with teichuronic acids[238] under phosphate limitation are representative examples in eubacteria and cyanobacteria. HSO_3-PtdGro in *H. mediterranei* (strain R-4) appears to be replaced by an equivalent increase in the amounts of HSO_3-6Man-2Glc-$E_{20}E_{20}$Gro (S-DGD).[326] The major barrier amphiphiles including phosphatidylglycerol in bacteria,[60,155] and sulfatides in halophilic archaea or vertebrates (for rev. ref. 238) increased in adaptation to high environmental osmolality. These anionic lipids arose most likely as the result of convergence of the structure in molecular evolution. Genes of the adaptive machinery can be supplied from phylogenetically widely separated species. A possible descendence of sialic acid-related and polygalacturonate-related genes from a common ancestor gene has been suggested.[501] The genes related to the sialic acid metabolism such as sialidase found in prokaryotes may have been transferred from the host animal via phages horizontally to bacteria.[474]

Lipophilic residues also seem to be replaceable.[238] The gram-negative rods of genus Sphingomonas contained GlcUCer[380] and a 'lipid A-type', glucosamine-containing tetrasaccharide ceramide instead of lipopolysaccharide (LPS) usually found in the gram-negative bacterial membrane.[280] The membrane fraction of *Sphingomonas paucimobilis*, the LPS-lacking gram-negative bacterium, contained glycosphingolipids that was assumed to have a function similar to that of the LPS of other gram-negative bacteria.[280] The hybrid structure of this LPS is interesting in two ways: (1) ceramide with cyclopropanic sphinganine linked to a saccharide containing glucosamine amide, which in turn, is linked to 2-hydroxymyristic acid instead of 3-hydroxymyristic acid in normal lipid A, can replace LPS; (2) phylogenetically, the genes related to the metabolism of ceramide must have been transferred from the eukaryote host. Even the presumably advantageous molecular device such as LPS that has survived a billion years was able to be substituted by a glycosphingolipid![659] The neutral theory dictates that the great majority of evolutionary changes at the molecular level are not the sequel of Darwinian selection but the consequence of the random fixation of selectively neutral[286] or very nearly neutral[440] alleles under continued mutation pressure. Farther examples of the neutral evolution of sulfatide structures and replaceability with other anionic lipids will be accumulated in future.[238]

VI. BIOSYNTHESIS AND BIODEGRADATION

In as early as 1960, [^{35}S] labeling of rat *in vivo* demonstrated that brain, kidney, and liver incorporated sulfate into lipid fractions.[24,151] Recently, a GalCer sulfotransferase was purified from human renal cell carcinoma,[211] and the cDNA was cloned.[210] On the other hand, the sulfate is released from sulfatides by a lysosomal soluble enzyme and the activator protein on the lysosomal membrane.[302]

A. Biosynthesis

1. In Vivo Studies

Labeling of the whole animal or intact cell with [^{35}S]sulfate or [^{35}S]methionine[24,382] and the chase of the incorporated glycolipid-[^{35}S]O_3^- reflected the turnover rate, the

difference between synthesis and degradation, of sulfatides. The turnover of sulfatides in the whole body of eels,[654] rats[24,151,233] and mice[53,171,608] was studied by intraperitoneal injection of [^{35}S]sulfate to animals. The correction for the age-dependent blood level was necessary for the quantitative estimation of the turnover rate because the blood [^{35}S]sulfate level decreased with time after injection of the isotope.[54]

The maximal incorporation of the isotope into rat brain lipids occurred 48 hr after the administration of radioactive sulfate, after which time the activity remained constant through the 16th day, but slowly fell thereafter; although on the 32nd day, the level was still 3/4 of that found on the second day[151] and the specific radioactivity of [^{35}S]SM4s in myelin decreased only slightly even after 197 days.[248] SM4g but not HSO_3-Chol was significantly labeled by [^{35}S]sulfate.[120,228] Incubation of rat brain slices with [^{35}S]sulfate labeled only SM4s[596] whereas rat sciatic nerve slices incorporated [^{35}S]-radioactivities into both SM4s, and SMGlcUnLc$_4$Cer.[68] The half-life of kidney sulfatides was considerably shorter than that of testis and myelin.[233] Intraperitoneal administration of [^{35}S]sulfate to rats labeled HSO_3-Chol,[233,559] SM4s, SM4s-Glc,[217] SM2a,[557] SB2,[558] and SB1a in kidneys.[561] In the testis of mice[171] or rat,[189,305,352,517] intraperitoneally administered [^{35}S]sulfate labeled only SM4g. [^{35}S]Sulfate injected intratesticularly,[119,306,341] or administered *per os*[582] into rat was also incorporated into SM4g in the testis whereas HSO_3-Chol was not significantly labeled. After incubation of guinea pig testicular slice in a medium containing [^{35}S]sulfate, autoradiography of the lipid fraction showed only SM4g.[545]

Primary cultures of animal cells and established cell lines are the convenient system for metabolic studies. Oligodendrocytes,[27] as well as Schwann cell lines[26,110] incorporated [^{35}S]sulfate into SM4s, SM4g and several sulfated gangliosides;[111] and melanoma cells into HSO_3-Chol, SM4s, SM3, SMUnLc$_4$Cer, and SMUnLc$_6$Cer.[469,583] Incubation with [^{35}S]sulfate of the renal tubular epithelial cell lines including MDCK,[49,234,423] JTC-12,[234,555] MDBK,[234,424] LLC-PK$_1$,[424] SMKT-R3 renal carcinoma cells[291,293] and Verots cell lines[425] labeled HSO_3-Chol, SM4s, SM3, and some sulfatides of the ganglio-series. Hepatocellular carcinoma cell lines, a cholangiocarcinoma cell line,[204] and endometrial adenocarcinoma cells[255,319] of humans incorporated [^{35}S]sulfate into SM4s and/or SM3. Photosynthetic bacteria[155] and myeloid cell lines[17] were cultured in a commercial sulfate-free medium (CRCM-30, Sigma), or a medium with the sulfate salts substituted with chloride salts, then labeled with carrier-free [^{35}S]sulfate. However, the results of sulfation in sulfate-deprived (carrier-free) medium should be evaluated carefully[611] since culture of cells using a sulfate deficient medium can result in undersulfation of glycosaminoglycans in some cell types[612] and sulfation of each chain is an 'all or nothing' process.

Dissociated primary cultures of rat brain were incubated with varying quantities of galactose with a fixed amount of radioactivity per culture.[521] In a medium containing 1 mM galactose, more than 85% of the label was present in the carbohydrate moiety of glycolipids.[521] When cells were incubated with [^{3}H]-hexose,[230] [^{14}C]hexose-, or [^{14}C]serine,[62] radioactivities were distributed in the fatty acid, sphingosine, and sugar moieties.

2. *Sulfotransferase*

Little has been reported on the properties of glycolipid sulfotransferase of halophilic archaea and mycobacteria.[149,273] The sulfotransferases of plant and animal cytosol sulfate flavonoids, steroids and other aryl compounds, but not glycolipids.[658] In photosynthetic prokaryotes and plant chloroplasts, 6-sulfoquinovose is transferred from UDP-6-sulfoquinovose to the position *sn*-3 of A_2Gro by the catalysis of UDP-sulfoquinovose: diacylglycerol sulfoquinovosyltransferase.[188] When the gene of this enzyme, which catalyzes the last step of SQ-A_2Gro synthesis of photosynthetic bacterium *Rhodobacter sphaeroides*, was inactivated, the resulting sulfolipid-deficient mutant accumulated the precursor UDP-sulfoquinovose.[155]

The *in vitro* formation of SM4s, via transfer of sulfate from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to GalCer catalyzed by a sulfotransferase, GalCer sulfotransferase (EC 2.8.2.11), has been demonstrated in brain,[108,361] kidney,[104,105,495] testis,[171,289,353] rat gastric mucosa,[377] lung,[138] human endometrium,[255,319] and sera from patients with renal cell carcinoma.[136,137]

The GalCer sulfotransferase activity was demonstrated in the Golgi-rich fraction from rat kidneys,[105] rat testis,[289] and a Schwann cell line.[110] The cytosolic sulfotransferases act on aryl compounds, hydroxysteroids, estrogens, and bile acids.[137,210] The localization of GalCer sulfotransferase at the lumen of Golgi apparatus is similar to that of glycosaminoglycan sulfotransferases and tyrosylprotein sulfotransferase. The site of sulfation may be localized to the distal Golgi or trans-Golgi network[110] because brefeldin, which destroys (or blocks vesicular transport) distal to medial Golgi, inhibited SM4s synthesis completely, while both hydroxy and nonhydroxy GalCer were synthesized. In contrast, 2-hydroxyceramide galactosyltransferase is thought to be located on the endoplasmic reticulum of myelinating rat brain,[510] and MDCK II cells.[51]

Some drugs affect the synthesis of sulfatides without modification of sulfotransferase. Incubation of the myeloid cell lines THP-1, and HL60,[17] or a human renal carcinoma cell line SMKT-R3[293] in the medium containing sodium selenate, an inhibitor of PAPS formation, resulted in a reduction of SM4s expression. Monensin, an inhibitor of vesicular transport from the medial- and trans-Golgi to the plasma membrane, also inhibited 75% of $[^{35}S]O_4^{2-}$ incorporation into sulfolipids.[617] Brefeldin blocks, in a reversible manner, the anterograde movement of vesicular traffic from the endoplasmic reticulum (ER) to the Golgi, allowing retrograde movement back to the ER to continue.[110]

Honke and his group[211] purified PAPS:GalCer sulfotransferase from a human renal cancer cell line SMKT-R3 through a combination of affinity chromatographies using GalSph, 3′,5′-bisphosphoadenosine and heparin as ligands.[211] The purified human renal sulfotransferase showed a highest specific activity of 18,000 μ mol/mg/hr[211] and a single protein band with an apparent molecular mass of 54 kDa. pH optima were between 6.5,[211] to 7.0[542] similar to other transverses of Golgi membrane. The putative hydrophobic transmembrane domain of GalCer sulfotransferase from human renal carcinoma cell contained 23 amino acid residues characteristic of type II transmembrane proteins. The cloned GalCer sulfotransferase showed homology neither to the cytosolic sulfotransferases nor to the Golgi glycosaminoglycan sulfotransferases.

3. Substrate Specificity

GalCer, that is abundant in mammalian oligodendrocytes, kidneys, and the gastrointestinal tract, was usually the best acceptor for GalCer sulfotransferase. LacCer,[109,171,555] Gal-EAGro,[171,487] and Gal-A_2Gro[211,289,463,586] were also good acceptors. GalSph,[586] GlcCer, Gg_4Cer, nLc_4Cer, Gg_3Cer, SM2a,[292] and Gb_4Cer did serve as acceptors although the relative activities were low.[211] The glycolipids with the terminal α-Gal in Galα-4GalCer[171,463] or Gb_3Cer,[171,211] and cholesterol[463] did not serve as acceptors. GlcCer appeared not to be the substrate of sulfotransferase in the brain of mice deficient in GalCer.[75] Absence of sulfatides with an internal *N*-acetylgalactosamine 3-sulfate suggests that GalNAc 3-sulfate might be a termination signal against further sulfation or glycosylation of the glyco-amphiphiles. On the other hand, oligosaccharides including galactose and lactose did not serve as substrates or a competitive inhibitor for the purified enzyme from the renal carcinoma cell.[211]

Competition studies suggested that a single enzyme in the boar testis,[171] as well as in cultured renal cells, MDCK, JTC-12,[555] and SMKT-R3,[293] sulfated GalCer and LacCer. Highly purified human renal sulfotransferase sulfated LacCer and Gal-EAGro rapidly establishing that a single enzyme synthesizes SM4s, SM4g, and SM3.[211] A rat brain sulfotransferase was highly specific to GlcUnLc$_4$Cer and the rate-limiting step of the synthesis of SMUnLc$_4$Cer was the activity of a GlcNAc transferase.[69,70] The UDP-*N*-acetylgalactosamine: SM3 *N*-acetylgalactosaminyltransferase activity of rat brain, which

synthesized SM2a, recognized both GM3 and SM3 as equally good acceptors.[230,402] This lack of stringency suggested that the GalNAc transferase step cannot be a rate-limiting step of glycolipid biosynthesis.

The optimal assay conditions for GalCer with nonhydroxy and hydroxy fatty acids, and LacCer were significantly different.[171,555] GalCer with hydroxy fatty acid, synthesized at the cytosolic leaflet of the endoplasmic reticulum of MDCK II cells, may be translocated to the luminal leaflet of the Golgi membrane whereas the activity to synthesize GlcCer and GalCer with nonhydroxy fatty acids may be located on the cytosolic leaflet of proximal Golgi.[51] A ceramide containing a 2-hydroxy fatty acid may be preferentially converted to GalCer, Ga_2Cer, and SM4s by renal distal tubular cell line MDCK and colon carcinoma Caco-2 cells.[657] In mouse brain, however, only one ceramide galactosyltransferase exists that catalyzes the synthesis of both nonhydroxy and hydroxy fatty acid GalCer.[75]

4. *Intracellular Traffic and Membrane Polarity*

The apical membrane of polarized cells is enriched in glycosphingolipids.[657] Luminal (apical), but not basolateral, membrane of transporting cells including MDCK was enriched in both phospholipids and glycolipids[340,655] including SM4s.[49] By transport experiments using Cer with shorter chain fatty acids, on the contrary, sphingomyelin, GalCer with 2-hydroxy fatty acids, LacCer and SM4s were transported preferentially to the basolateral cell surface when compared to GlcCer.[657] When immature rat eyes were incubated in a medium containing [^{35}S]sulfate, there was a rapid *in vitro* uptake of the label into SM4s of the ciliary processes, the site of active transport.[32] Electron microscopic autoradiography showed that initially (7 min-label) the label was localized in the apical cytoplasm where the Golgi apparatus is located but with longer periods (30–60 min-chase) of incubation in nonradioactive medium it moved to the infolded basal and lateral plasma membrane.

Exogenous glycosphingolipids are incorporated into the plasma membrane[128] or internalized by endocytosis,[386,655] packed in a vesicle and sent to lysosomes.[128] The fluorescent analogue of SM4s, *N*-lissamine rhodaminyl-(12-aminododecanoyl)-SM4s (LRh-SM4s)-albumin complexes (and not the fluorescent SM4s alone) was transferred into the plasma membrane by the cell. In order to incorporate glycolipids into SMKT-R3 cells, SM4s dissolved in a small amount of dimethylsulfoxide was exogenously added to the cells.[328] Cytofluorometry showed that the incorporation of SM4s into the cells considerably increased the reactivity with Sulph-I and laminins in a dose-dependent manner.[294] LRh-SM4s complexed to albumin were used to study the metabolic fate of SM4s in oligodendrocytes under microscope.[386,617] The degradation at 24 hr-pulse reached 18% in normal fibroblasts, whereas it was not detectable in metachromatic leukodystrophy cells. An exogenous [^{3}H]labeled ceramide with hydroxy fatty acids adsorbed on BSA was preferentially incorporated into GalCer and SM4s in comparison to a ceramide with nonhydroxy fatty acids.[657]

B. *Regulation*

Lipophilic and hydrophilic hormones modify the expression and activities of sulfotransferases by the interplay with nuclear receptors and promoters of genes, and transmembrane cascades respectively.

1. *Lipophilic Hormones*

Incorporation of [^{35}S]sulfate into HSO_3-Chol fraction of rabbit tracheal epithelial cells increased 50–100-fold upon differentiation, suggesting that the accumulation of HSO_3-Chol can be a marker of differentiation of tracheal cells.[464] This accumulation was the result of induction of cholesterol sulfotransferase and was completely blocked by the in-

clusion of retinoic acid analogues, the inhibitor of squamous differentiation, in the culture medium.[464] Progesterone in association with estradiol, stimulated the synthesis of HSO_3-Chol in rabbit endometrium[384] and HSO_3-Chol and hydrophobic sulfated conjugates in subcultured glandular epithelial cells of guinea-pig endometrium.[422]

Reduction of the level of SM4g to approx. 70% of the normal testis in hypophysectomized rats may reflect the suppression of spermatogenesis concomitant with the decrease in androgens of both adrenocortical and testicular origin.[306] Retinoid (Vitamin A) deficiency also caused degeneration of seminiferous tubules and results in sterility of the rat. SM4g in the testes of rat fed a retinol-deficient diet for 46 days decreased to 13% of the control rats fed a retinol-deficient diet for 20 days and then supplemented with 140 μg/rat/day of retinol palmitate for 26 days.[546] Vitamin K treatment for 3 days significantly enhanced brain sulfotransferase activities, whereas administration of the vitamin K antagonist, Warfarin, drastically reduced the enzyme in 2 weeks.[541]

The thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), are essential for the maturation of oligodendrocytes and play a stimulatory role in myelination of the central nervous system. The rate of synthesis of sulfatides remained drastically diminished throughout a 70-day developmental period when brain cells from embryonic mice were grown in the presence of hypothyroid calf serum,[36] while the activity was restored to normal levels after 72 hr of exposure to a medium supplemented with exogenous T_3. In the presence of T_3, the incorporation of [^{35}S]sulfate into sulfatides (SM4s and SM4g) of oligodendroglial cultures obtained from the brains of 1-week-old rats exhibited a developmental profile which is comparable to that found in the developing brain *in vivo*[304] whereas omission of T_3 resulted in lower rates of sulfatide synthesis. Incorporation of [^{35}S]sulfate into SM4s fraction of glioma cells, G26–19 and C26–20, was stimulated up to 2–6-fold in the presence of 5×10^{-6} M cortisol or dexamethasone in culture media with the concomitant 3–4-fold stimulation of sulfotransferase activities.[86,250] Experiments with cycloheximide and actinomycin D showed that the effect of the hormone on glycolipid synthesis on these cells was mediated through de novo mRNA and protein synthesis.[250] Testosterone, androsterone, and estradiol had no stimulatory effect.

2. *Water-soluble Hormones and Growth Factors*

[^{35}S]sulfate incorporation into HSO_3-Chol increased in JTC-12 and MDCK cell lines by additions of butyrate to culture medium.[233] The incorporation into SM2a and GM2 in JTC-12 cells was also stimulated by [^{35}S]labeling *in vivo* depending on the dose of butyrate.[556] Only a trace amount of radioactivity was incorporated normally into SM3 of MDBK cell line, whereas by additions of butyrate (2.5 mM), the incorporation into SM3 increased 20-fold. By incubation of the culture of C_6 glioma cell line with 50 mM disipramine, a tricyclic antidepressant, SM4s synthesis was substantially stimulated but HSO_3-Chol level was unchanged.[592] Prosaposin, saposin C, and peptides (prosaptides), encompassing the neurotrophic sequence located in the saposin C domain, also increased SM4s concentrations in primary and transformed Schwann cells and oligodendrocytes.[201]

When growth factors of tyrosine kinase type, transforming growth factor α (TGF-α) (0–50 ng/ml[290]),[25] epidermal growth factor (EGF),[290] and hepatic growth factor (HGF, 5–50 ng/ml),[291] were supplemented to the medium of the culture of SMKT-R3 cells, the sulfotransferase activity was increased markedly (approx. 300%). The incorporation of [^{35}S]sulfate into MDCK cells increased by renewal of the medium with 10% serum supplemented by hyperosmolar NaCl.[426] On the other hand, supplementation of hypertonic NaCl after 2 days of serum depletion resulted in an increase of sulfotransferase corresponding to approx. 50% of that with the simultaneous addition of serum.[231,443] Both of these increases were canceled by cycloheximide, an inhibitor of protein synthesis, suggesting that the synthesis of some proteins was responsible for the increased synthesis of sulfatides.[291,426] On the contrary, removal of serum from the culture of mouse oligodendroglioma cells resulted in approx. 2-fold enhancement of the sulfatide levels and

$H_2[^{35}S]O_4$ incorporation into SM4s within 24 hr with concomitant increase of sulfotransferase activity.[250] Tyrosine kinase inhibitors, genistein and tyrphostin 51, reduced the enhancement of the sulfotransferase activity by EGF in a dose- and time-dependent manner.[25] Polycystic kidney disease is a disorder marked by aberrant renal tubular epithelial cell proliferation and transport abnormalities in *cpk/cpk* mice.[93] GlcCer, LacCer, and GM3 (V.C.1) displayed a large increase in the kidney of 3-week-old *cpk/cpk* mice, while SM4s and Cer concentrations decreased with concomitant decrease of GalCer sulfotransferase.

3. *Sulfatide Modulation of Enzyme Activities*

SM4s increased the activity of phospholipase C on the dilauroylphosphatidylcholine monolayer at various surface pressures[38] and activated proteinkinase C to the same extent as phosphatidylserine did with phorbol esters.[129] HSO_3-Chol activated η-, ε-, and ζ-isoforms of proteinkinase C *in vitro* to an even greater extent.[92,220] On the other hand, SM4s and HSO_3-Chol, as well as some detergents inhibited the activity of phosphatidylinositol-3-kinase.[633] Exogenously added SM4s and anti-L-selectin antibodies enhanced tyrosine phosphorylation of MAP kinase in neutrophils.[622] The tyrosine kinase inhibitor, genistein, blocked the transient increase in intracellular Ca^{2+} and oxidative burst induced by SM4s, suggesting that this tyrosine phosphorylation serves to mediate signal transduction. Chymotrypsin-like activity of multicatalytic proteinase purified from human erythrocytes was selectively activated 2.5–3.5-fold by sulfated amphiphiles (SM4s, SM3, and SDS) but not by neutral glycolipids, gangliosides, and HSO_3-Chol. Heparin also activated the trypsin-like activity 2.5-fold, while other mucopolysaccharides did not.[438]

Sulfatides (SM4s, SM4g, 5 μM), and gangliosides (50 μM) inhibited the activity of DNA polymerase α, but acidic phospholipids did not.[439,520] This inhibition of the enzyme activity by acidic glycolipids was suppressed by nonionic detergents suggesting that these acidic glycolipids, in the form of micelles, exerted influence on the enzyme activity by behaving as a polyanionic macromolecule.[439] Human spleen lysosomal enzymes, glucocerebrosidases, were activated by acidic lipids (0.3–4.7 mM) including phosphatidylserine, a ganglioside, SM4s, synthetic monoacylglycerol sulfates and diacylglycerol sulfates.[143]

C. *Cerebroside Sulfatase and Saposins*

Arylsulfatase A (EC 3.1.6.1) has been detected in most prokaryotes, plants, and the lysosome of animal tissues.[106] Only GalCer sulfatase (EC 3.1.6.8), which is the same enzyme as arylsulfatase A (EC 3.1.6.1), is responsible for cleavage of the sulfate esters of sulfatides. Sulfatides are not hydrolyzed by the other lysosomal enzymes arylsulfatase B (EC 3.1.6.12), and glucosamine-6-sulfatase which desulfate glycosaminoglycans. The third arylsulfatase, arylsulfatase C (EC 3.1.6.2), is in endoplasmic reticulum and desulfates steroid sulfates.[502] Arylsulfatase A catalyzes the cleavage of *O*—S bond similar to the acid hydrolysis of sulfoconjugates.[482]

$$R \cdot O \cdot SO_3^- + H_2O \rightarrow R \cdot OH + H^+ + SO_4^{2-}$$

Treatment of glucose 3-sulfate with arylsulfatase A in $H_2{}^{18}O$ resulted in the formation of $S^{18}O^{16}O_3^{2-}$, supporting the cleavage of the *O*—S bond.[601]

Arylsulfatases from alimentary tract of *Helix pomatia* were used for enzymatic desulfation of sulfatides immobilized on a 96-well plate.[139,204] SM4s and SM4g were desulfated by an arylsulfatase fraction from the hepatopancreas of a mollusc *Charonia lampas*. The sulfate ester of these sulfatides was, however, scarcely hydrolyzed by 'glycosulfatase' I and II (EC 3.1.6.3), purified from the same source, which had been known to hydrolyze the sulfate ester linkage at C6 of glucose.[189] The acidic forms of the arylsulfatase from

various invertebrates possessed SM4s sulfohydrolase activities,[389] whereas the arylsulfatase A fraction from ox liver showed 'glycosulfatase' activity.[482] The mRNA of arylsulfatase of sea urchin embryos reached a peak during development between the 32-cell stage and the prism stage.[660]

Mammalian arylsulfatase A hydrolyzes sulfate esters of sulfatides including SM4s,[361] SM4g,[119,134,135,641] lyso-SM4s (HSO_3-3GalSph),[99] ascorbic acid 2-sulfate, and tyrosine-*O*-sulfate[106] but not SM4s-6,[374] SMUnLc$_4$Cer and SMUnLc$_6$Cer.[68] The human arylsulfatase A gene was about 3.2 kb long and contained eight exons.[140,313] Multiple transcription initiation sites were located between nucleotides −367 and −387 of this housekeeping gene, although its expression remained constant with EGF, TGFα or HGF treatment.[291] The genes coding for human arylsulfatase A, human steroid sulfatase, human glucosamine-6-sulfatase, and an arylsulfatase from sea urchin contained substantial amounts of homologous DNA sequences and probably originated from a common ancestral gene.[502]

Sphingolipid activator proteins (sap) or *saposins* are water-soluble, and heat-stable glycoproteins of about 1 kDa (80 amino acids) which interact with sphingolipids with oligosaccharide chains up to a length of three hexoses.[128,393,492] The cDNA of prosaposin gene, the presumed 'housekeeping' gene located at chromosome 10q21 in humans, codes for a sequence of 524 amino acid residues in four domains (A, B, C and D).[540] Probably alternative splicing of mRNA[330] and post-translational proteolytic processing[196] generate four small, homologous proteins. *In situ* hybridization showed that prosaposin mRNA was expressed differentially in cell types and at developmental stages, and synthesized prosaposin was targeted via the mannose 6-phosphate receptor system to the lysosome or released into body fluids including serum, milk and semen.[540] Saposins and saposin-like proteins contain three intradomain disulfide linkages, which create a common structural framework upon which amino acids in four amphipathic alpha helices can carry out diverse functions including transport of glycolipids.[393]

Saposin B (sap-B or SAP-1), a homodimeric glycoprotein, has a relatively wide specificity and interacts with GalCer, SM4s, sphingomyelin, phosphatidylserine, Gb$_3$Cer, Gg$_4$Cer, Ga$_2$Cer, as well as gangliosides GM1, GM2 and GM3[531] (Table 2). However, SM4s, but not Gb$_3$Cer and GM1 ganglioside, was accumulated in sap-B deficiency, suggesting that the physiological role of sap-B is solubilization of sulfatides.[393,508] Deglycosylated sap-B interacted with SM4s and GM1 ganglioside identical to native sap-B and stimulated the enzymatic hydrolysis of SM4s by arylsulfatase A to the same extent as native sap-B.[203] Another saposin, glucosylceramidase-activator sap-C (SAP-2), and two additional potential activator proteins (sap-A and sap-D) are derived from a common precursor prosaposin by proteolytic processing. Sap-B, localized on lysosomal membrane, extracts a single sulfatide molecule from micelles or membranes probably behaving as a physiological detergent and binds it as a water-soluble 1:1 complex.[128] Deacylation of SM4s and SM4g (lyso-derivatives) or substitution of the fatty acid with acetic acid resulted in hydrolysis of the sulfate without a saposin probably because they form small micelles.[115,616]

Sulfatides are accumulated in metachromatic leukodystrophy (MLD) patients' myelin, liver, gallbladder, pancreatic islet cells, anterior pituitary, adrenal cortex (Table 3), and sweat glands.[302] MLD can be caused by mutations in two different genes, the arylsulfatase A[436] and the prosaposin genes.[197] Patients with a complete defect of the prosaposin gene (due to a mutation in the initiation codon) exhibited complex biochemical abnormalities.[197] Mutant mice homozygous for an inactivated prosaposin gene exhibited two distinct clinical phenotypes, neonatally fatal and later-onset, with accumulation of glycolipids including SM4s.[130] X-linked ichthyosis is a genetic defect of the X chromosome with steroid sulfatase deficiency resulting in increased HSO_3-Chol in serum and stratum corneum giving rise to shedding of large scales containing a 5-fold increased HSO_3-Chol.[630,661] Multiple sulfatase deficiency (MSD) is an inborn error of metabolism with deficiency of arylsulfatase A, B, and C accompanied with accumulation of SM4s, SM3, HSO_3-Chol and heparan sulfate in the central nervous system, kidney and liver.[502,661]

The co- or posttranslational conversion of a cysteine (Cys69 and Cys91 in arylsulfatase A, and B respectively) to 2-amino-3-oxopropionic acid appeared to be required for generating catalytically active sulfatases.[502]

GM2 activator (SAP-3) stimulates the release of the terminal GalNAc from GM2, Gg_3Cer, SM2a,[230] and Gb_4Cer by β-hexosaminidase A by binding with the substrate.[164] Microbial activator of endoglycoceramidase did not catalyze hydrolysis of SM4s or cerebrosides.[240]

VII. INTERACTION WITH BIOMOLECULES

Sulfatides interact with various biomolecules specifically participating in cell adhesion, differentiation, and signal transduction.[611]

A. Electrostatic Interactions

As early as 1960, the high-affinity interaction of various neurotransmitters to SM4s was reported.[152,153] A particularly strong stereospecific and electrostatic bond can be formed between the protonated nitrogen of opioids and the sulfate group of SM4s[356] or anionic phospholipids.[4] Azure A (2×10^{-7} M) inhibited 85% of the specific electrostatic interaction of [^{3}H]morphine and [^{3}H]naloxone to synaptosomal membrane.[356] Generally, proteins that interact with heparin also interact with fucoidan, dextran sulfate or sulfatides. The positively charged sequence (KKNKED) in L-selectin interacted with acidic phospholipids and fucoidan[363] and sequences rich in basic amino acids are contained in the domains responsible to SM4s binding in thrombospondin and laminin. Tyrosine sulfate residues may also be able to cooperate with immediately adjacent sialylated oligosaccharides to generate P-selectin recognition of PSGL-1 (P-selectin glycoprotein ligand-1).[345] In this instance the clustered patch may not be purely made up of saccharides, but would be a composite 'clustered anionic patch'.[80] From this point of view, most of the affinity of sulfated glycoconjugates with antibodies or 'specific' binding proteins[61] may involve, primarily, ionic interactions.

In situ hybridization showed the colocalization of the mRNA of ceramide galactosyl transferase and myelin basic protein (MBP).[510] The developmental expression pattern was also similar to the myelination profile.[533] It has been proposed that SM4s, together with other amphipathic compounds, interacts with hydrophobicity[528] or electrostatic force[493] with MBP, which contains predominantly hydrophobic and basic amino acids, stabilizing the compact structure of myelin. However, the physiological significance of this phenomenon has been questioned because MBP is an extrinsic membrane protein associated to the cytoplasmic leaflet of the bilayer and a direct interaction with SM4s at the exoplasmic leaflet is unlikely.[618] Due to its highly basic amino acid composition, Tamm–Horsfall glycoprotein (T–H) of the kidney, a glycosylphosphatidylinositol anchored protein, has been a potential candidate for ionic interaction with SM4s. Studies by using human T–H[648] and polyclonal antibodies specific to SM4s[649] showed that T–H and SM4s had a strictly superimposable localization on kidney tissue sections, that is at the luminal membrane of the thick ascending limb of the loop of Henle and the initial portion of the distal convoluted tubule beyond the macula densa. In view of the presence of 3-*O*-sulfated galactose in T–H, however, attention has to be paid to the possibility that antibodies recognizing 3-*O*-sulfated galactosyl residues may also cross-react with T–H.[185] Serum amyloid-P protein has been known to interact with a variety of anionic amphiphiles including hexose phosphates in the presence of Ca^{2+}. Amyloid-P interacted preferentially with sulfatides with sulfate groups on the terminal Gal or GalNAc residues, whereas there was considerably weaker interaction with SM2a that has a sulfated penultimate Gal, and only trace binding to SM1a with an internal sulfated Gal.[357]

B. Proteins of the Extracellular Matrix and Blood

The extracellular matrix proteins including laminins, fibronectins and collagens, as well as plasma proteins including thrombospondins, have multidomain structures containing interaction sites for various types of anionic glycoconjugates including heparin and sulfatides[611] (Table 2). For laminin interaction with sulfatides including SM4s, SM4g, SM3,[294,471] SMUnLc$_4$Cer and SMUnLc$_6$Cer,[249,381,503] a sulfate group at the non-reducing terminus is necessary. Eight genetically distinct laminin chains (α1, α2, α3, α4, α5, β1, β2, β3, γ1, γ2) and ten different heterotrimeric assembly forms, laminins -1 to -10, are known so far. The major isoform, laminin-1, has a distinct affinity for heparin and SM4s mediated by fragment E3, which consists of the G4 and G5 domains of the carboxyl terminus of α1 chain with two X-B-B-X-B-X, and three B-X-B-X-B-X sequences.[583,591] Urea treatment or reduction and alkylation of the fragments abolished SM4s binding[583] but not the binding to heparin and HNK-1 neoglycoproteins[163] suggesting that sulfatide interaction specifically requires an intact three-dimensional structure. In contrast to SM4s and heparin, glycoconjugates with SMUnLc$_4$ appeared to bind to E-8 fragment of the domain G2 of laminin-1.[163]

Thrombospondins (TSP) are large, homotrimeric glycoproteins.[43] TSP1 and TSP2 contain a globular NH_2-terminal domain, a procollagen homology domain, type I (TSP or properdin), and type II (EGF-like), and type III (Ca^{2+}-binding) repeats, and a carboxyl terminal domain.[43] Thrombospondins interacted on solid phase with SM4s, SM3, SM4g,[43] SM2a, SMGb$_4$Cer,[400] and SMGb$_5$Cer[399] from human kidney. Human properdin, and H-Factor, the regulators of the alternative pathway of complement activation, interacted with SM4g, SM4s, SM3, SB2, and SM2a.[246] A consensus sequence for the interaction with heparin and SM4s was proposed as Cys-Ser-Val-Thr-Cys-Gly-X-Gly-X-X-X-Arg-X-Arg (or -Lys) (=CSVTCGXGXXXRXR).[207–209] TSP type I repeats, properdin (a serum protein),[198] antistasin (a leech salivary anticoagulant), and *Herpes simplex* I contain these motives. The coat protein,[62,390,446] region II-plus of the[390] circumsporozoite (CS) proteins, and thrombospondin-related anonymous protein (TRAP) of malaria parasites also contain an amino acid motif based around the sequence CSVTCG.[585] This amino acid motif has been considered to confer on the CS protein the ability to bind specifically to host sulfated glycoconjugates and to the surface proteoglycans of hepatocytes or HepG2 cells.[390,522] CSVTCG has also been suggested to be an important determinant in interaction with other mammalian cells,[43] although it was shown recently that the downstream positively-charged residues without CSVTCG or WSPWS segment served as the potent ligand.[667]

Heparin and sulfated glycolipids had a stronger affinity to WSXW sequence in peptides obtained from thrombospondin, laminin, and apolipoprotein.[157] The heparin-binding domains of TSP1 and 2 were subsequently shown to contain BBXB sequences (e.g. KRFK), where B is the probability of a basic residue and X is a hydropathic residue, and the type I repeat contains WSXW in addition to CSVTCG. However, TSP type I repeat 5 of properdin contains neither of the electroneutral sequences, the WSXW or the CSVTCG motif, while rich in basic amino acids.[198] XBBXB and XBBBXXBX were determined as the consensus sequences for glycosaminoglycan recognition in 49 regions in 21 proteins.[61]

It has been proposed that soluble laminins in the culture medium mediate adhesion of some melanoma cell lines to sulfatides (SM4s or SM4g, 25 fmol/mm^2) on the plastic plate.[472] The interaction of ^{125}I-labeled laminin or thrombospondin to A2058 melanoma cells was inhibited by thrombospondin peptides containing WSXW sequence.[157] The peptide KRFKQDGGWSHWSPWSS inhibited binding by approx. 80–95%. Many other tumor cell lines have been found to attach on SM4s substrates using endogenous proteins interacting with SM4s (Table 2).

C. Selectins

All three (E-, P-, and L-) selectins possess the *N*-terminal carbohydrate-recognition domain homologous to C-lectins (Ca^{2+}-dependent vertebrate lectins) followed by an EGF-like domain, and recognize sialyl-Lewis x and its isomer sialyl-Lewis a.[613] L- and P-selectins also interact with diverse natural sulfoglycolipids and sulfated neoglycolipids in Ca^{2+}-dependent[23,112,301,467] or independent[153,363,417,550] modes (Table 2).

A soluble fusion protein of rat L-selectin-IgG, which contained the lectin and EGF domains,[550] was assayed for the ligands by solid-phase binding assay. L-selectin-IgG interacted with sulfated glycolipids in the concentrations of 30–100 pmol/well. A synthetic SM4s analogue, HSO_3-3Gal-B30 (branched chain, C30 alcohol) interacted more preferentially with the chimera protein than HSO_3-2Gal-B30, or HSO_3-6Gal-B30. Also, $(HSO_3)_2$-3,6Gal-B30 and $(HSO_3)_3$-3,4,6Gal-B30 were more reactive than HSO_3-3Gal-B30, indicating that the interaction of L-selectin with its sulfated sugar ligands is position-specific but depends on the number of the sulfate group supporting the role of anion clusters for the interaction.[393,550] Soluble chimeric proteins with various domains switched between E- and L-selectins showed that the *N*-terminal lectin domain bound specifically to SM4s,[301] while EGF domain or the consensus repeats of E- and L-selectins had no influence on the specificity to carbohydrates.[301] In contrast, conservative substitution of Ser-124 and 128 in EGF domain residue was able to alter E-selectin binding such that it adhered to SM4s and heparin, and reduced P-selectin adherence to these ligands.[467] The binding site for acidic phospholipids is located close to the lectin domain and contained a BBXBXX (i.e. KKNKED) sequence.[363]

By the use of rat L-selectin-IgG chimera, ligands for L-selectin were located in high endothelial cells in lymph nodes, the white matter, neurons, cerebellar Purkinje cells, and choroid plexus of the central nervous system, as well as in a straight portion of distal tubules and capillary blood vessels in medulla and pelvis of the kidney.[581] The staining on the white matter of the cerebellum and distal tubules of the kidney was completely abolished by treatment with organic solvents (C/M, 1:1), indicating that the major ligands for L-selectin-IgG chimera in these organs were glycolipids. However, the true, high-affinity ligand at the high-endothelial venules (HEV) may be diverse mucins with sialylated, sulfated, fucosylated lactosamine-type *O*-linked.[80]

Using computer modeling techniques, site-specific mutagenesis, and ligand and cell binding assays, it was shown that P-selectin lectin domains bind to myeloid cells, SM4s and sialyl Lewis x oligosaccharide via an overlapping, but not identical set of residues located in a shallow cleft of the lectin domain that is similar to the mannose-binding protein saccharide-binding site proximal to a functional calcium binding site.[23,467] When Ala77 was substituted with lysine, P-selectin-carbohydrate binding specificity changed from sialyl Lewis x to oligomannose supporting the structural analogy with the lectin domain of rat mannose-binding protein.[467]

D. Microorganisms

Sulfatides interact with several viri including HIV (human immunodeficiency virus), cytomegalovirus[435] and influenza virus.[549] The ability of the surface envelope glycoproteins, gp120[656] and GP41[460] from different strains of HIV to interact with MAG, sulfatides or acidic phospholipids has been regarded as an important determinant in the development of neuropathy of AIDS, although CD4 molecule is the primary ligand for HIV.[663] GalCer and SM4s were stained with ^{125}I-labeled gp120 but GlcCer, GM1, GD1a and neutral glycolipids from human erythrocytes were not.[37] In another experiment using nitrocellulose paper, however, gp120 bound only to SM4s and not to GalCer.[662] The interaction with a peptide of V3 domain of gp120 suggested that the GalCer and SM4s binding region may reside on the sequence of the V3 loop peptide.[406] Fully sulfated Glc, Gal and lactose linked to lipophilic moieties,[219] the sulfated polysaccharides (e.g. curdlan sulfate),[276] SQ-A_2Gro, as well as medium-molecular-weight sulfated alkyl

oligosaccharides[148] showed anti-HIV activities *in vitro*. By sulfated alkyl laminara- and malto-oligosaccharides (0.4 μg/ml) a 50% decrease of HIV-induced cytopathic effects was observed.[276,277] The infection of human T cells with the virus was markedly inhibited by treatment with the sulfated gangliosides at a concentration of 10 μg/ml, while the non-sulfated gangliosides had only weak antiviral activities.[168]

The cell adhesion to sulfatides immobilized on TLC plates[506] demonstrated that many prokaryotes use glycoconjugates as the receptors.[263] S-fimbriated *E. coli* strains, the pathogens of neonatal meningitis, interacted with SM4s and SM4g, but the transformants which lack the sfaA gene showed no interaction with the glycolipids.[454] *M. pneumoniae* bound avidly to pulmonary tissues as well as the WiDr human colon adenocarcinoma cell line, and also interacted on TLC plates with SM4s, SM4g and SM3,[314,470] while *M. hyopneumoniae* interacted with SM4s, GM3 and Gb_4Cer.[652] *Mycoplasma pulmonis* is associated with male infertility in humans, cattle and rodents probably by binding specifically to SM4g.[354] The topology of mycoplasma interaction with rat sperm was consistent with the known topology of sperm SM4g.[355] Moreover, dextran sulfate and heparin[354,444] inhibited the interaction between Mycoplasmas and cells or tissues that express SM4s or SM4g.

SM4s showed strong tetanus-toxin binding on HPTLC plates, with standard GD1b and GT1b as positive controls.[144] The absence of tetanus-toxin-binding gangliosides in SCLC (small-cell-lung-cancer) cell lines suggested that sulfatides might be responsible for the reaction of SCLC cells with the toxin. Sulfated glycolipids may serve as the mucosal receptor for colonization of *Helicobacter pylori*, which colonizes only in the gastric epithelium and is associated with gastritis and peptic ulcer.[412] Viable *H. pylori* showed a strong interaction with SM4s, SM3 and GM3 by TLC-immunostaining.[259,404] Virulent strains of *B. pertussis*, the human respiratory pathogens, bound specifically to asialo GM1 and SM4s, suggesting that the *B. pertussis* adhesin FHA may utilize sulfated glycolipids and proteoglycans to initiate infection.[48,172]

VIII. PERSPECTIVES

The status of the present efforts to establish functions specific to sulfatides is more or less analogous to the trends in sulfated polysaccharides of the connective tissue described by Comper[76] and in sulfated glycoconjugates reviewed by Varki.[611] The functional roles assigned to the anionic proteoglycans and polysaccharides of cell surface materials of prokaryotes or extracellular matrix of multicellular organisms contain a great insight applicable to sulfated glycolipids. The partial informational specificity of sulfated glycoconjugates has been discussed in terms of evolutionary flexibility of the extracellular matrix.[76] Matthews indicated, as early as 1975, that the response to environmental influences is an essential property of supporting tissues and that this characteristic is derived largely from the capacity for *fine modulation* of the structure of its constituent macromolecules.[367] To name the major tools for the gross modulation against hyperosmolality, they are transporters such as Na^+,K^+-ATPase, and organic osmolytes including glucitol, glycerol, *myo*-inisotiol, betain, and glycerylphosphorylcholine.[358,410] As much evidence has suggested, the multi-step fine modulation may be accomplished by anionic polysaccharides and sulfated glycolipids forming multilayers on the cell surface.[426]

The recent results obtained by inactivation of the genes of sugar transferases in knockout mice were quite unexpected and some of them appeared to suggest the redundancy of anionic glycolipids in the biosphere. For instance, the mice with the inactivated gene of β-GalNAc transferase that transfers β-GalNAc at position 4 of the Gal in LacCer survived with unexpectedly slight growth retardation. In the above cases, GM3 and GD3[132] replaced 'conventional' ganglio-series gangliosides in agreement with the neutral theory of glycolipid structure,[238] and allometric principle.[398] The major part of the function of GalCer and sulfatides in myelin appeared to be partly replaceable by other amphiphiles as shown by the knockout mice with inactivated ceramide galactosyltransferase.[75] However, the serious defect of these mice suggested that the replacement is not adequate

for the full expression of the myelin function. The biological molecules in the biosphere have either been evolutionally selected for their ability to adapt the environment or at least partly resulted from neutral diversification and random drift.[238]

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